STUDIES ON S-ALLELE INCOMPATIBILITY IN BRASSICA OLERACEA

Margaret Sedgley

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STUDIES ON S-ALLELE INCOMPATIBILITY

IN

BRASSICA OLERACEA

by

MARGARET SEDGLEY

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TABLE OF CONTENTS.

LIST OF TABLES.

LIST OF GRAPHS.

LIST OF DIAGRAMS.

LIST OF PHOTOGRAPHS.

Following page 181.

Number of photograph

1. Double diffusion plate, well pattern 1.
   Well 1. As. 8, 17.7.72, abs. 3:1, 2. $S_{45}S_{45}$,
   3. $S_{45}S_{2}$, 4. $S_{45}S_{23}$, 5. $S_{16}S_{16}$, 6. $S_{45}S_{45}$,
   7. $S_{2}S_{2}$, $S_{5}S_{5}$ & $S_{15}S_{15}$. 1% agar gel.

2. As 1 but 0.5% agarose gel.

3. Double diffusion plate, well pattern 6.
   Well 1. $S_{16}S_{16}$, 2. As. 10, 13.6.72.
   3. As. 10, 13.6.72, 1/2, 4. As. 10, 13.6.72, 1/4,
   5. As. 10, 13.6.72, 1/8. 8 ml 0.5% agarose gel.

4. Double diffusion plate, well pattern 6.
   Well 1. $S_{16}S_{16}$, 2. As. 10, 13.6.72, 1/16,
   3. As. 10, 13.6.72, 1/32, 4. As. 10, 13.6.72,
   1/64, 5. As. 10, 13.6.72, 1/128. 8 ml 0.5%
   agarose gel.

5. As 3 but 10 ml 0.5% agarose gel.

6. As 4 but 10 ml 0.5% agarose gel.

7. Double diffusion plate, well pattern 2.
   Well 1. $S_{45}S_{45}$, 2. As. 8, 2.2.72, 3. As. 8,
   2.2.72, 1/2, 4. As. 8, 2.2.72, 1/4, 5. As. 8,
   2.2.72, 1/8, 6. As. 8, 2.2.72, 1/16,

7. As. 8, 2.2.72, 1/32. Saline extraction of stigmas.

8. As 7 but phosphate/saline buffer extraction of stigmas.

9. Double diffusion plate. Upper wells $S_{45}S_{45}$, lower wells As. 8, 2.2.72. Comparison of well spacings.

10. Double diffusion plate, well pattern 1.
Well 1. As. 2, 8.7.71, 2. $S_{2}S_{2}$, 3. $S_{45}S_{45}$,
4. $S_{15}S_{15}$, 5. $S_{2}S_{2}$, 6. $S_{45}S_{45}$, 7. $S_{15}S_{15}$.
2-4 fresh extract, 5-7 frozen extract.

11. Double diffusion plate, well pattern 1.
Well 1. Nasrallah As. 5, abs. 3:1, 2. $S_{SD7}S_{SD7}$,
3. $S_{45}S_{45}$, 4. $S_{SD7}S_{2}$, 5. $S_{46}S_{46}$,
6. $S_{SD7}S_{SD7}$, fresh extracts.

12. As 11 but frozen extracts.

13. Double diffusion plate, well pattern 2.
Well 1. $S_{23}S_{23}$, 2. As. 6, 15.9.71,
3. As. 6, 15.9.71, 1/2, 4. As. 6, 15.9.71, 1/4,
5. As. 6, 15.9.71, 1/8, 6. As.6, 15.9.71, 1/16,
7. As. 6, 15.9.71, 1/32. Before centrifuging of stigma extract.

14. As 13 but after centrifuging of stigma extract.

15. As 13 but after freesing of stigma extract.

16. Double diffusion plate, well pattern 1.
Well 1. As. 12, 4.5.72, abs. 3:1, 2. S_5S_5,
3. S_5S_5, 4. S_2S_2, 5. $S_{15}S_{15}$, 6. S_5S_5,
7. $S_{23}S_{23}$, S_2S_2 & $S_{45}S_{45}$. Before freesing
of absorbed serum.

17. As 16 but after freesing of absorbed serum.

18. Double diffusion plate, well pattern 1.
Well 1. As. 6, 3.3.72, Abs. 3:1, 2. $S_{23}S_{23}$,
3. $S_{23}S_{23}$, 4. S_8S_8 & $S_{25}S_{25}$, 5. S_8S_8,
6. $S_{23}S_{23}$, 7. $S_{23}S_{23}$. Before concentration
of absorbed serum.

19. As 18 but after concentration of absorbed serum.

20. Double diffusion plate, well pattern 1.
Well 1. Nasrallah As. 5, abs. 3:1,
2. $S_{SD7}S_{SD7}$, 3. $S_{SD7}S_{SD7}$, 4. $S_{SD7}S_{SD7}$,
5. S_2S_2, 6. $S_{SD7}S_{SD7}$, 7. S_2S_2.

21. Double diffusion plate, well pattern 1.
Well 1. Nasrallah As. 5, abs. 3:1,
2. $S_{SD7}S_{SD7}$, 3. $S_{SD7}S_{SD7}$, 1/2, 4. $S_{SD7}S_{SD7}^{1/4}$,
5. $S_{SD7}S_{SD7}^{1/8}$, 6. $S_{SD7}S_{SD7}^{1/16}$.

22. Double diffusion plate, well pattern 1.
Well 1. Nasrallah As. 5, abs. 3:1,

2. $S_{SD7}S_{SD7}$ 1/32, 3. $S_{SD7}S_{SD7}$ 1/64, 4. $S_{SD7}S_{SD7}$ 1/128, 5. $S_{SD7}S_{SD7}$ 1/256, 6. $S_{SD7}S_{SD7}$ 1/512.

23. Double diffusion plate, well pattern 1. Well 1. Nasrallah As. 5, abs. 3:1, 2. $S_{SD7}S_2$, 3. $S_{SD7}S_2$ 1/2, 4. $S_{SD7}S_2$ 1/4, 5. $S_{SD7}S_2$ 1/8, 6. $S_{SD7}S_2$ 1/16.

24. Double diffusion plate, well pattern 4. Well 1. As. 2, 8.7.71, abs. 3:1, 2. S_2S_2, 3. S_2S_2, 4. S_2S_2, 5. $S_{45}S_{45}$.

25. Double diffusion plate, well pattern 1. Well 1. As. 2, 8.7.71, 2. S_2S_2, 3. S_2S_2, 4. $S_{45}S_{45}$, 5. $S_{45}S_2$, 6. S_2S_2, 7. $S_{45}S_{45}$. All stigma extracts diluted by 1/2.

26. As 25 but well pattern 5.

27. Double diffusion plate, well pattern 3. Well 1. As, 2, 8.7.71, abs. 6:1, 2. S_2S_2, 3. S_2S_2, 4. S_2S_2, 5. $S_{45}S_{45}$.

28. As 27 but well pattern 4.

29. Double diffusion plate, well pattern 1. Well 1. As. 3, 26.7.71, 2. $S_{15}S_{15}$, 3. $S_{15}S_{15}$, 4. $S_{15}S_{45}$, 5. $S_{15}S_{15}$, 6. $S_{16}S_{16}$, 7. S_2S_2.

30. As 29 but serum absorbed 4:1.

31. As 29 but serum absorbed 10:1.

32. As 29 but serum diluted 5:1.

33. Double diffusion plate, well pattern 3. Well 1. As. 5, 24.8.71, abs. 3:1, 2. $S_{23}S_{23}$, 3. $S_{23}S_{23}$, 4. $S_{27}S_{27}$, 5. $S_{16}S_{16}$, 6. $S_{23}S_{23}$, 7. $S_{2}S_{45}$.

34. Double diffusion plate, well pattern 3. Well 1. As. 6, 15.9.71, 2. $S_{2}S_{2}$, 3. $S_{15}S_{15}$, 4. $S_{23}S_{23}$, 5, $S_{45}S_{45}$, 6. $S_{16}S_{16}$, 7. $S_{27}S_{27}$.

35. As 34 but As. 6, 20.10.71.

36. Double diffusion plate, well pattern 3. Well 1. As. 6, 25.11.71, 2. $S_{15}S_{15}$, $S_{16}S_{16}$ & $S_{27}S_{27}$, 3. $S_{23}S_{23}$, 4. $S_{2}S_{45}$ & $S_{2}S_{2}$.

37. Double diffusion plate, well pattern 3. Well 1. As. 6, 6.12.71, 2. $S_{23}S_{23}$, 3. $S_{15}S_{15}$, 4. $S_{27}S_{27}$, 5. $S_{16}S_{16}$, 6. $S_{15}S_{15}$, 7. $S_{2}S_{45}$.

38. Double diffusion plate, well pattern 3. Well 1. As. 6, 6.12.71, 2. $S_{23}S_{23}$, 3. $S_{27}S_{27}$, 4. $S_{2}S_{45}$, 5. $S_{23}S_{26}$, 6. $S_{23}S_{23}$.

39. Double diffusion plate, well pattern 3.

Well 1. As. 6, 15.9.71, abs. 3:1, 2. $S_{23}S_{23}$, 3. $S_{16}S_{16}$, 4. $S_{23}S_{23}$, 5. $S_{23}S_{23}$, 6. $S_{27}S_{27}$, 7. $S_{23}S_{23}$.

40. Double diffusion plate, well pattern 1. Well 1. As. 6, 25.11.71, abs. 3:1, 2. $S_{23}S_{23}$, 3. $S_{23}S_{45}$, 4. $S_{25}S_{25}$ & $S_{8}S_{8}$, 5. $S_{8}S_{8}$, 6. $S_{17}S_{17}$, 7. $S_{23}S_{23}$.

41. As 40 but As. 6, 6.12.71, abs. 3:1.

42. As 40 but As. 6, 23.2.72, abs. 3:1.

43. As 40 but As. 6, 14-21.12.71, abs. 3:1 & Well 3. $S_{23}S_{23}$.

44. As 43 but As. 6, 25.2.72, abs. 3:1.

45. As 40 but As. 6, 25.11.71.

46. As 40 but As. 6, 6.12.71.

47. As 40 but As. 6, 21.2.72.

48. As 40 but As. 6, 23.2.72.

49. As 43 but As. 6, 14-21.12.71.

50. As 43 but As. 6, 25.2.72.

51. Immunoelectrophoresis plate. As. 6, 14-21.12.71. Upper well $S_{23}S_{23}$, middle well S_8S_8, $S_{17}S_{17}$ & $S_{25}S_{25}$, lower well $S_{23}S_{23}$. Before freezing of stigma extracts. Cathode right, anode left.

52. As 51 but As. 6, 25.11.71.

53. As 51. After freezing of stigma extracts.

54. Double diffusion plate, well pattern 2. Well 1. As. 7, 16.9.71, 2. $S_{23}S_{23}$p. dilute, 3. $S_{23}S_{23}$p. concentrated, 4. $S_{23}S_{23}$s.

55. Double diffusion plate, well pattern 3. Well 1. As. 7, 16.9.71, 2. $S_{23}S_{23}$p., 3. $S_{23}S_{23}$s., 4. $S_{23}S_{23}$p., 5. $S_{23}S_{23}$ s.

56. As 55 but As. 6, 15.9.71.

57. Double diffusion plate, well pattern 1. Well 1. As. 7, 20.9.71, 2. $S_{23}S_{23}$p., 3. $S_{23}S_{23}$ s. open flowers, 4. $S_{23}S_{23}$ s. buds, 5. $S_{23}S_{23}$ p.

58. As 57 but As. 6, 14-21.12.71.

59. Double diffusion plate, well pattern 1. Well 1. As. 7, 20.9.71, 2. $S_{23}S_{23}$ p., 3. $S_{23}S_{23}$ p. 1/2, 4. $S_{23}S_{23}$ p. 1/4, 5. $S_{23}S_{23}$ p. 1/8, 6. $S_{23}S_{23}$ p. 1/16, 7. $S_{23}S_{23}$s.

60. As 59 but well pattern 2.

61. Double diffusion plate, well pattern 3.
Well 1. As. 7, 16.9.71, 2. $S_{23}S_{23}$ p., 3. $S_{23}S_{45}$ p.,
4. $S_{23}S_{23}$ p., 5. $S_{23}S_{23}$ s., 5. $S_{16}S_{16}$ s.,
7. $S_{45}S_{45}$ s.

62. As 61 but well pattern 1 & As. 7, 16.9.71,
abs. 3:1.

63. Double diffusion plate, well pattern 1.
Well 1. As. 7, 20.9.71, 2. $S_{23}S_{23}$ p.
200,000 Moon units/ml., 3. $S_{23}S_{23}$ p. diffusate,
100,000 Moon units/ml., 4. $S_{23}S_{23}$ p. 1/2,
100,000 Noon units/ml., 5. $S_{23}S_{23}$ p. diffusate,
100,000 Moon units/ml.

64. As 63 but well pattern 2.

65. Double diffusion plate, well pattern 1.
Well 1. As. 8, 31.1.72. - 4.2.72, 2. $S_{45}S_{45}$,
3. S_5S_5, $S_{15}S_{23}$ & S_2S_2, 4. $S_{45}S_{45}$, 5. $S_{45}S_{45}$.

66. As 65 but As. 8, 31.1.72. - 4.2.72, abs. 3:1 &
Well 6. S_5S_5, $S_{15}S_{23}$ & S_2S_2 (30 stigmas in 0.1
mls.)

67. Double diffusion plate, well pattern 3.
Well 1. As. 9, 26.11.71, 2. $S_{27}S_{27}$, $S_{16}S_{16}$ &
S_5S_5, 3. $S_{23}S_{23}$, 4. $S_{45}S_2$ & S_2S_2.

68. As 40 but As. 9, 14.12.71.

69. Double diffusion plate, well pattern 1.
Well 1. As. 10, 16.5.72, abs. 3:2.5, 2. $S_{16}S_{16}$, 3. $S_{14}S_{14}$, 4. $S_{2}S_{2}$, $S_{5}S_{5}$ & $S_{21}S_{21}$, 5. $S_{16}S_{16}$, 6. $S_{23}S_{23}$, 7. $S_{16}S_{16}$.

70. Double diffusion plate, well pattern 6.
Well 1. $S_{16}S_{16}$, 2. As. 10, 11.4.72, abs. 3:1 3. As. 10, 11.4.72, abs. 3:1, 1/2, 4. As. 10, 11.4.72, abs. 3:1, 1/4, 5. As. 10, 11.4.72. abs. 3:1 1/8.

71. Double diffusion plate, well pattern 1.
Well 1. As. 10, 9.5.72, abs. 3:1.5, 2. $S_{16}S_{16}$, 3. $S_{5}S_{5}$, 4. $S_{23}S_{23}$, $S_{2}S_{2}$, $S_{27}S_{27}$ & $S_{15}S_{15}$, 5. $S_{16}S_{16}$, 6. $S_{21}S_{21}$, 7. $S_{16}S_{16}$.

72. As 69 but As. 10, 23.5.72, abs. 1:1.

73. As 69 but As. 10, 31.5.72, abs. 1:1.

74. As 69 but As. 10, 6.6.72, abs. 1:1.

75. As 69 but As. 10, 13.6.72, abs. 3:1.

76. Double diffusion plate, well pattern 6.
Well 1. $S_{16}S_{16}$, 2. As. 10, 31.5.72. abs. 1:1, 3. As. 10, 31.5.72, abs. 1:1, 1/2, 4. As. 10, 31.5.72, abs. 1:1, 1/4, 5. As. 10, 31.5.72,

abs. 3:1, 1/8.

77. As 76 but serum absorbed with a mixture containing $S_{14}S_{14}$.

78. Double diffusion plate, well pattern 1. Well 1. As. 10, 31.5.72, abs. 1:1, 2. $S_{16}S_{16}$, s., 3. $S_{16}S_{16}$P. diffusate, 4. $S_{16}S_{16}$P. diffusate 1/2, 5. $S_{16}S_{16}$P. diffusate 1/4, 6. $S_{16}S_{16}$s. 7. $S_{16}S_{16}$P.

79. Double diffusion plate, well pattern 1. Well 1. As. 10, 31.5.72, abs. 1:1, 2. $S_{16}S_{16}$, 3. $S_{16}S_{16}$saline diffusate, 4. $S_{16}S_{16}$Coca's fluid diffusate, 5. $S_{16}S_{16}$.

80. Double diffusion plate, well pattern 1. Well 1. As. 10, 31.5.72, abs. 1:1, 2. Gelatin caps from 10 $S_{16}S_{16}$ stigmas, 3. $S_{16}S_{16}$, 4. $S_{16}S_{16}$ 1/2, 5. $S_{16}S_{16}$ 1/4, 6. Gelatin caps from 10 $S_{16}S_{16}$ stigmas, 7. Gelatin caps from 10 matchstalks.

81. Double diffusion plate, well pattern 1. Well 1. As. 11, 12.4.72, abs. 3:1, 2. S_2S_2, 3. S_2S_2, 4. $S_{45}S_{45}$, 5. $S_{45}S_{45}$, 6. S_2S_2, 7. $S_{15}S_{15}$, $S_{45}S_{23}$, S_5S_5 & $S_{23}S_{23}$.

82. As 81 but As. 11, 19.4.72, abs. 3:1.

83. As 81 but As. 11, 26.4.72, abs. 3:1 & 4.
S_2S_{45}.

84. As 83.

85. As 83 but As. 11, 3.5.72, abs. 3:1.

86. As 81 but As. 11, 10.5.72, abs. 3:1.

87. As 86 but different absorbing stigmas.

88. As 87 but As. 11, 17.5.72, abs. 3:1.

89. Double diffusion plate, well pattern 1.
Well 1. As. 12, 27.4.72, abs. 3:1, 2. S_5S_5,
3. S_5S_5, 4. S_2S_2, 5. $S_{15}S_{15}$, 6. S_5S_5,
7. $S_{23}S_{23}$, $S_{23}S_{45}$ & $S_{45}S_{45}$.

90. As 16 but As. 12, 18.5.72, abs. 3:1.

91. Double diffusion plate, well pattern 1.
Well 1. As. 5, 3.4.72, abs. 3:1, 2. $S_{23}S_{23}$,
3. $S_{23}S_{45}$, 4. S_5S_5, 5. $S_{23}S_{23}$, 6. S_2S_2,
$S_{27}S_{27}$, $S_{16}S_{16}$ & $S_{21}S_{21}$, 7. $S_{23}S_{15}$.

92. Immunoelectrophoresis plate. As. 5, 17.4.72.
Upper well $S_{23}S_{23}$, centre well S_8S_8,
$S_{17}S_{17}$ & $S_{25}S_{25}$ & lower well $S_{23}S_{23}$.
Cathode left, anode right.

93. Double diffusion plate, well pattern 1. Well 1. As. 8, 17.7.72, 2. $S_{45}S_{45}$. 3. $S_{45}S_{45}$, 4. S_2S_2, 5. S_5S_5, S_2S_2 & $S_{15}S_{15}$, 6. $S_{45}S_{45}$, 7. S_5S_5.

94. Double diffusion plate, well pattern 1. Well 1. As. 8, 3.7.72, abs. 3:1, 2. $S_{23}S_{23}$, S_2S_2 & S_5S_5, 3. $S_{16}S_{16}$, 4. $S_{45}S_{45}$, 5. $S_{45}S_{45}$, 6. $S_{23}S_{45}$, 7. $S_{45}S_{45}$.

95. As 94 but As. 8, 10.7.72, abs. 3:1.

96. As 93 but As. 8, 17.7.72, abs. 3:1.

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DECLARATION

I hereby declare that the thesis which follows is my own composition, that it is a record of work done by myself and that it has not been presented in any previous application for a higher degree.

CERTIFICATE

I certify that Margaret Sedgley, a candidate for the Degree of Doctor of Philosophy in the Unversity of St. Andrews, has fulfilled the conditions of the University Court Ordinance No. 16, so that she is qualified to submit her thesis.

SUMMARY.

By the end of 1972, a total of thirteen rabbits had been injected. One serum was raised to *Brassica oleracea* $S_{23}S_{23}$ kale pollen proteins, but no S-antibody was stimulated. All the others were raised to stigma proteins. A range of S-alleles and varieties of *B.oleracea* was used including $S_{23}S_{23}$, $S_{16}S_{16}$ and $S_{14}S_{14}$ kale, S_2S_2, $S_{45}S_{45}$ and S_5S_5 brussels sprout and $S_{15}S_{15}$ cabbage. S_{15} and S_5 were of low dominance, S_2 and S_{45} of intermediate dominance and S_{23}, S_{14} and S_{16} of high dominance. S-antibodies were raised to S_{23} and S_{16} in kale, and S_2 and S_{45} in brussels sprout. All were of intermediate or high dominance. Only the S_{16}-antibody had a high titre of 1/16, the others were 1/4 or less. In two of the cases, booster injections stimulated the S-antibody response where the first course of injections had failed. Relatively dilute stigma extracts of 250 stigmas/ml stimulated most S-antibodies. An injection schedule of more than two months produced cross-reactions or new specificities. Extract which had been frozen or treated with formalin gave a poorer antibody response than freshly-prepared extract. No S-antibodies were stimulated by these treatments. In its present form, the technique was unsuitable for routine S-allele diagnosis in *B.oleracea* because of the low rate of successful sera and the low titres stimulated. Improvements to the technique are suggested.

Between 35 and 40% of the total protein content was lost when a stigma extract was frozen, but the S-protein was not lost.

B.oleracea pollen germinated on an agar and sucrose medium. Germination was stimulated by 0.0005% quercetin, but tube growth was not. Quercetin was detected as glycosides in the pollen and stigma tissue of *B.oleracea*. It was not involved in the incompatibility reaction of the stigma.

List of abbreviations.

b.s.	brussels sprout
c.	cabbage
k.	kale
k/bs.	kale/brussels sprout cross.
i/m	intra-muscular
i/v	intra-venous
s/c	sub-cutaneous
N.	normal solution
M.	molar solution
uv.	ultra violet
C.	Californian
N.Z.W.	New Zealand White
fls.	flowers
No.	number
inj.	injection
concn.	concentration
ab.	antibody
as.	antiserum
abs.	absorbed
p.	pollen
s.	stigma
S.H.R.I.	Scottish Horticultural Research Institute

INTRODUCTION.

The demand for uniform crops of brussels sprout, cabbage and kale, especially for once-over mechanical harvesting, has led to the introduction of F_1 varieties. To produce these varieties, plants are selfed over a number of generations to develop self-incompatible inbred lines which are as homozygous as possible. Bud pollination is used to produce selfed inbred seed as the incompatibility barrier is not built up until one or two days before the flower opens. Plants of two such inbred lines chosen for their capacity to combine together well to produce a vigorous uniform hybrid with good crop characteristics, are planted together in alternate rows. This seed crop must be isolated from all other flowering *Brassica oleracea* plants to prevent contamination by foreign pollen. In practice, the resultant F_1 seed contains a proportion of selfed progeny of one or both of the parents. This is probably due partly to selection for partially self compatible individuals during the generations of inbreeding, and partly to environmental conditions which influence self incompatibility during seed set, such as high humidity (Tatebe 1964). The selfed parents in a hybrid crop are known as "sibs".

The F_1 technique is not the only one used to produce hybrid *Brassica* varieties. Thompson 1959 has described a double-cross, and in 1964 a triple-cross method of producing hybrid kale. In the double-cross method, the two F_1 progeny derived from four inbreds are used as the crop parents, and in the triple-cross, the two F_1 progeny are each crossed with a different inbred and the resulting progeny used as the crop parents. The production of synthetic varieties is also being considered for Brassica breeding. A synthetic variety is one which is maintained from open pollinated seed following its synthesis by hybridisation in all combinations among a number of selected genotypes (Allard, 1960). The genotypes that are hybridised would be inbred lines in the case of Brassica, and, as in the single, double and triple cross hybrid methods, the S-allele composition of the inbreds must be known to be different before a successful variety can be produced.

At present, cross-compatibility is tested by pollination. After the pollinations have been carried out, compatibility can be assessed by seed production, but this takes several weeks before results are obtained. Alternatively, the pollinated styles can be removed from the plant after 24 hours, fixed, softened, stained with aniline blue and observed using a microscope illuminated by ultra violet light. (du Crehu, 1968, van Hal and Verhoeven 1968). This method stains the callose in the pollen tubes which fluoresces under ultra violet light. It is much quicker than the seed set method as results can be obtained within 72 hours.

The aim of this study was to assess the serological techniques described by Nasrallah and Wallace 1967 a & b for use as a routine diagnostic S-allele test. An antiserum containing an S-protein-specific antibody, when absorbed, could be used to identify a stigma extract from a plant containing the S-allele to which the antiserum had been raised. If antisera could be raised to all the S-alleles in the breeding material then each plant could be typed as to its S-allele status. This would reduce the time and effort involved in S-allele identification even further as a few stigmas, some gels and a little absorbed serum to each of the likely S-alleles is all that would be needed to identify the S-genotype of a plant. Results could be obtained within 24 hours, without the effort involved in pollination, staining and microscopy.

Nasrallah and Wallace 1967 a & b, used serological techniques to detect a protein thought to be associated with the incompatibility reaction of B.oleracea. Lewis, 1952, had detected such a protein in the pollen of Oenothera organensis and Linskens 1960, had obtained similar results with the pollen and style tissue of Petunia hybrida. B.oleracea, which includes cabbage, as used by Nasrallah, but also kale and brussels sprout as used in this study, has a sporophytic and multiallelic incompatibility system which exhibits dominance relationships (Thompson and Taylor 1966a). The barrier to self-pollen and pollen containing an S-allele in common with the receptor plant, is the stigma and this was the tissue used for most tests, both by Nasrallah and in this study.

An extract of stigma tissue was prepared and used for injection into a rabbit to stimulate the production of antibodies to the protein constituents of the extract by the immune system of the rabbit. After a course of injections, the serum from the rabbit would cause specific precipitation of the proteins in the stigma extract where before the injections it would not. This precipitation could be seen more clearly in a gel medium through which the stigma extract and serum could diffuse freely. Agar is such a medium and between two wells in an agar gel, one charged with serum and one with extract, a series of precipitation bands could be seen, each representing a protein which had stimulated its own specific antibody in the rabbit's body. This is referred to as double diffusion, as both reactants diffuse. The different positions of the bands were due to differences in size and mobility of the proteins and antibodies. Nasrallah found, as previously shown by Lewis, that if a sample of serum raised in response to stigma extract from one plant of a certain S-allele constitution, was mixed with a stigma extract from another plant, as far as possible identical with the first plant except in its S-alleles, then precipitation of all but one protein occurred. The one protein remaining unprecipitated was that associated with the S-allele constitution of the plant as this was the only protein difference between the two plants. This was called the 'S-protein'. Thus, when the wells in the agar were charged with stigma extract and absorbed serum, only one band was visible and this represented the S-protein of the S-allele involved.

During the summer of 1970 Dr. Nasrallah spent three months at the Scottish Horticultural Research Institute and raised six antisera in six rabbits. On his departure he left a little of each serum and tests were carried out on one which was found to contain an S-protein-specific antibody.

During 1971, when the work described here was commenced, nine rabbits were injected and eight antisera were raised. All rabbits were of the Californian breed. Seven antisera were raised to stigma extracts and one to a pollen extract. Brussels sprout, cabbage and kale plants homozygous for their S-alleles were used as sources of stigma tissue; four alleles were involved, S_{23} (high dominance), S_{15} (low dominance) and S_{45} and S_2 (intermediate dominance). The dominance classifications were determined by Thompson and Taylor 1966a and Thompson, unpublished data. Marrow-stem kale carrying S_{23} provided the pollen source. These antisera were numbered one to nine.

In 1972 a further four antisera were raised and numbered ten to thirteen. More work was carried out involving rabbits 5, 6 and 8. Rabbits of New Zealand White breed were compared with the Californians; two of each were used in 1972. The new antisera were raised to stigma tissues homozygous for the following S-alleles; S_{16} and S_{14} (high dominance), S_2 (intermediate dominance) and S_5 (low dominance). Brussels sprout and kale plants were involved.

CHAPTER I.

LITERATURE SURVEY.

Self-incompatibility is a mechanism whereby a plant fails to produce selfed seeds although its gametes are functional. Incompatibility systems can be classified as heteromorphic or homomorphic. With heteromorphic incompatibility, different flower forms which inhibit or reduce self-pollination are produced on plants of the same species. Within the homomorphic type, genetic mechanisms control pollen behaviour which can be gametophytic or sporophytic. In the former the behaviour of the pollen grain is governed by its own haploid genotype, in the latter it is governed by the diploid genotype of the Parent plant. *B.oleracea* belongs to the homomorphic group with sporophytic pollen control. Plants with S-allele incompatibility systems are also cross-incompatible with plants containing the same functional S-allele. Self-incompatibility occurs widely among flowering plants and has been found in at least 71 families and 250 genera (Brewbaker 1959). It is a mechanism which promotes outbreeding. Within the Cruciferae, which is the family to which *B.oleracea* belongs, there are 102 self-compatible and 80 self-incompatible species. (Bateman 1951).

The first report of the occurrence of self-incompatibility in plants was made by Kølreuter in 1764. He noticed that during two years, three plants of *Verbascum phoeniceum* would set no seed with their own good pollen, but would set seed with pollen from four other *Verbascum* species. For many years the phenomenon was known as self-sterility, and Sprengel 1793 explained it in terms of natural selection against self-pollination and fertilisation. Herbert 1837 found self-sterility in some *Hippeastrum* hybrids and in *Zephyranthes carinata*, and further instances were subsequently reported by many workers in a variety of species and genera.

Darwin 1876 was the first to investigate the Cruciferae in this context. He found that B.oleracea was partially self-compatible, setting about four times as much seed on outcrossing as on selfing. *Raphanus sativus* he found to be slightly less self compatible than *B.oleracea*, and *Iberis umbellata* and *I.amara* he found to be highly self-fertile. The complete self-sterility which he found in other genera he interpreted from the point of view of natural selection against inbreeding, as did Sprengel. He was unaware of cross-sterility and believed that pollen from any other plant of the same species would fertilise a self-sterile individual. Hildebrand 1896, also studied the Cruciferae, and found *Hesperis tristis*, *Lobularia maritima (Alyssum maritimum)*, *Cardamine pratensis*, *Rapistrum rugosum*, *Iberis pinnata* and *Sobolewskia clavata* to be fully self-sterile. *Aethionema grandiflorum* and *Hugueninia tanacetifolia (Nasturtium tanacetifolium)* he found to be nearly self-sterile, and *Draba verna* and *Brassica rapa* were fully self-fertile.

In 1907, Jost proposed that each plant contains its own "individualstoffe" which stimulates the pollen of other plants but not its own. This was similar to Darwin's idea as universal cross-fertility within a species was still thought to occur. Jost also drew attention to the similarity between self-sterility in plants and the immunity reaction of animals which was later to be put forward by East 1929 as a theory to explain self-sterility. From his microscope examinations of Corydalis cava, C.lutea, Secale cereale, Cytisus laburnum and Lupinus albus, Jost was the first to record that the growth rate of a pollen tube in a sterile cross was much slower than in a compatible cross.

The first genetical interpretation was put forward by Correns 1912 to explain self-sterility in the Crucifer Cardamine pratensis. He applied Mendelian inheritance by assuming the existence of two factors, each of which inhibits the growth of pollen tubes from like gametes. To explain some self-compatible individuals, however, he assumed that the double recessive was self-fertile. This interpretation did not explain all Corren's results satisfactorily and he was not convinced that it was correct. It is interesting to note that the hypothesis assumed sporophytic control of the pollen and style reactions with independence and dominance. Compton 1913 regarded self-fertility in Reseda odorata to be a simple Mendelian dominant to self-sterility. He agreed with Jost's interpretation of an individual substance in each plant and similarity of self-sterility to the immunity reaction.

East 1915, disagreed with the simple Mendelian interpretations of Correns and Compton, although he did not offer an alternative explanation at this stage.

He did put forward a hypothesis, however, which suggested that a hexose sugar in the style was a pollen stimulator. The pollen contained an enzyme which differed slightly from plant to plant. This enzyme could "call forth secretion" of sugar in other plants but not in the plant from which it originated. In 1917, however, East and Park produced the first report of cross-sterility associated with self-sterility in *Nicotiana*. The pollen was assumed to act sporophytically at this stage (East 1917, 1918) and end-of-season fertility was noticed. Also in 1917, Sirks pointed out that incompatibility is not a form of sterility, and Stout 1917 was the first to use the term "incompatibility".

Between 1919 and 1926, various workers independently put forward the theory of oppositional factors. This proposed a series of multiple alleles, any two of which may be present in the style of a self-incompatible plant. Pollen is stimulated to grow only if it contains an allele different from those in the style. The first to put forward this theory was Lehmann 1919 and 1922, followed by Prell 1921, East and Mangelsdorf 1925 and Filzer 1926. This theory involved gametophytic control of the pollen reaction and was soon shown to apply to many species and genera.

In 1920, Stout found *Raphanus sativus* to be self-incompatible and in 1926 Detjen reported that some cabbage (*B.oleracea*) plants were self-incompatible but cross-compatible. Pearson 1929 secured data which led him to believe that sterility in cabbage was conditioned by a system of incompatibility factors.

Pearson also used bud fertility to produce selfed seed. The first report of this phenomenon was made by East in 1923 who found that unopened buds on self-incompatible Nicotiana plants would set seed with pollen from the same plant. Apparently, the incompatibility barrier was not set up until a day or so before bud burst. Pseudocompatibility or partial self-compatibility was known to occur within the Cruciferae. Stout 1922 reported on its occurrence in Brassica pekinensis and B.chinensis and over seven generations was unable to select a line of B.pekinensis showing higher self-compatibility than the pseudocompatible parents. (Stout 1927). Beatus 1929, 1931, repeated Correns' work with Cardamine pratensis but his results were confused by a high level of pseudocompatibility. He considered, however, that incompatibility was governed by oppositional factors of the Nicotiana-Veronica type, (Beatus 1934). Breiger 1927 and East 1929 considered that pseudo-compatibilty was governed by modifier genes. Also in 1929 East put forward his immunity theory to explain self-incompatibility, proposing that the selfed tubes secrete antigens which stimulate the style to produce antibodies which prevent further tube growth. This theory gained wide popularity as did the oppositional factors hypothesis with gametophytic control.

Kakizaki 1930 was the first to propose a genetical interpretation of incompatibility in B.oleracea. He proposed two series of alleles, one inhibitory and the other sympathetic. Both were assumed to act at the gametophytic level in the pollen as a result of

East's work on Nicotiana. The sympathetic series of alleles was proposed to account for self-compatibility, although the high level of pseudocompatibility encountered was probably classed as full self-compatibility (Bateman 1955). Also in 1930, Lawrence attempted to explain some of Correns' results on the oppositional factor hypothesis by assuming allotetraploidy of the material thus with four incompatibility factors. Correns' hypothesis was then disregarded. Working on B.pekinensis, Stout 1931 agreed with Kakizaki's interpretation and proposed the existence of fundamental factors also which were homozygous for a particular species. As a result of his microscope examinations of compatible and incompatible crosses he reported that in an incompatible cross there was a lower percentage of germinated grains and that those which did germinate often coiled around the stigmatic papillae rather than growing straight into the stigma.

Riley 1932, 1936 worked with the Crucifer Capsella and proposed a system involving two incompatibility loci with one gene epistatic to the other and sporophytic control of the pollen reaction. Capsella was subsequently cited as anomalous genus due to the sporophytic pollen control. However, Sears 1937 working with brocolli, B.oleracea var. italica and Raphanus sativus proposed two series of oppositional factors of varying inhibitory potency. Gametophytic pollen control was assumed. Sears also pollinated mutilated stigmas and found that compatibility resulted if part or all of the stigma was removed, but that slicing half way through the stigma with a razor in numerous planes parallel to the length of the style did not affect incompatibility. He concluded that the barrier to the pollen resided in the cell contents rather than in the cell wall. Tatebe 1939 repeated some of Sears' experiments and confirmed that removal of half or all of the stigma resulted in seed set. In 1944 Tatebe reported that a sporophytic system with alleles at two loci explained incompatibility in Japanese radish.

In 1950, Odland and Noll, and Attia and Munger both working with cabbage, _B.oleracea_, proposed oppositional S-alleles at one locus with gametophytic control of pollen behaviour. Both considered F_1 hybrid seed production for cabbage, using inbreds with different S-alleles and high combining ability. In the same year, Hughes and Babcock put forward that one incompatibility gene with multiple alleles which exhibit dominance and act sporophytically in the pollen governed incompatibility in _Crepis foetida rhoeadifolia_. This was also put forward by Gerstel 1950 to explain incompatibility in _Parthenium argentatum_. In 1953, however, Mizushima and Katsuo came to the same conclusion as Sears 1937 that incompatibility in cabbage was governed by oppositional factors at two loci with gametophytic control of pollen behaviour. In 1954, Crowe found that the hypothesis of Hughes and Babcock applied to _Cosmos bipinnatus_. She found dominance in the style as well as in the pollen and showed that the dominance sequence was not identical in pollen and style.

Bateman 1954 was the first to show that the single locus multiple series of alleles with sporophytic pollen control applied to a member of the Cruciferae. He worked with Iberis amara and showed that the two alleles of a heterozygote may act independently, or one may be dominant over the other. With certain pairs of alleles one of the pair was weakened though still active. Dominance and independence were found in both pollen and style and the relationships were not the same in the two. The alleles could be arranged in a linear order, slightly different for pollen and style. He estimated that the number of alleles was large, probably greater than 22. In 1955, Bateman reviewed the previously published data on incompatibility in the Crucifers and found that the results on Cardamine pratensis (Correns 1912), Capsella grandiflora (Riley 1932, 1936) and B.oleracea (Kakizaki 1930) were in agreement with those on Iberis, as well as some unpublished data on Raphanus sativus and B.campestris. In 1957, Thompson and Sampson independently published data on B.oleracea showing that Bateman was correct in his interpretation of the previously-published data. Further support came from Odland 1962 and Haruta 1962 and in 1957a, Sampson confirmed that the system applied to Raphanus sativus.

It was thus established that incompatibility in B.oleracea is governed by a series of multiple alleles at a single locus, with sporophytic control of pollen behaviour. Dominance relationships occur in both pollen and stigma and were confirmed to be non-linear by Thompson and Taylor 1966a. Sampson 1964 had previously reported non-linear dominance in the stigma of Raphanus raphanistrum. Thompson and Taylor 1966a determined dominance relationships for nearly a third of the possible combinations between the 28 alleles isolated from three varieties of kale. They found that non-linear dominance occurs more frequently in the stigma but that independent action is more common than dominance in pollen and stigma. Non-linear dominance was more frequent with alleles fairly low down in the dominance series. Thompson and Taylor 1966b found that the pollen-recessive alleles S_2, S_5 and S_{15} were common in botanical varieties of B.oleracea particularly the allele S_2. They also found that high frequencies of recessive S-alleles in a population are often associated with a higher proportion of partially and completely self-compatible plants. This they attributed to intensive selection by plant breeders for morphological and physiological characters. Over forty alleles have now been isolated by Thompson, they were numbered in chronological order of recognition (Thompson, unpublished data).

The gametophytic system of incompatibility is easier than the sporophytic to investigate from the physiological point of view as the pollen tube grows some way down the style before it is inhibited. The genetical mechanism was determined sooner for the gametophytic than for the sporophytic and for these two reasons more work has been carried out on the former. Emerson in 1940 showed that keeping a plant of Oenothera organensis in the dark weakened the self-incompatibility mechanism and Lewis 1942 showed that high temperatures caused increased inhibition of incompatible pollen *Oenothera*, *Prunus* and *Primula*. In 1943 Lewis suggested that the incompatibility substances are probably complex molecules whose specificity depends on the pattern of "determinant groups" attached to the basic molecule. The action of each S-allele produces a different pattern of determinant groups. The same author in 1949 pointed out that the time of S-gene action in the sporophytic system must be before or during early meiosis, whereas in the gametophytic it is after or during late Meiosis, but not earlier than telophase 1.

In 1953, Linskens showed that in *Petunia*, glucoprotein compounds in the conductive tissue differed in a compatible and incompatible cross. Since the compounds were not present at all in an unpollinated style, Linskens 1954 concluded that the complexes were products of a specific mutual reaction between style and pollen tube. Tracer experiments (Linskens 1958 and 1959) showed that both sexes contributed to these "ward bodies". Styles which had been self-pollinated also had a higher respiration rate than those which had been crossed (Linskens 1955) showing a metabolic disturbance. Electron micrographs of pollen tubes of *Petunia* showed denser cellulose membranes in selfed tubes (Mühlethaler and Linskens 1956) and in 1957, Linskens and Esser showed that the number of callose plugs was increased after pollen tube inhibition. Schlösser 1961, also working with *Petunia* produced further evidence of metabolic disturbance during self pollination by showing an increase in the activity of cytochrome oxidase and phosphatases and abnormal behaviour of the nuclei in an inhibited pollen tube.

Tupy 1961 found changes in the level of free amino acids in the styles of *Nicotiana alata* during pollen tube growth, the changes being greater after a compatible pollination. As a result of further work on *Nicotiana* (Tupy 1965), *Petunia* (Linskens and Tupy 1966) and *Oenothera* (Linder and Linskens 1972) it was concluded that most of the free amino acid was used for metabolic purposes during the growth of the pollen tube through the style. This may have included synthesis of incompatibility substances, but this has not been confirmed.

Brewbaker 1957 pointed out the correlation between pollen cytology and the place of inhibition in plants having incompatibility mechanisms. Plants showing gametophytic control have binucleate pollen and inhibition occurs in the style. Those with sporophytic control have trinucleate pollen which is inhibited at the stigma. Since trinucleate pollen has undergone a second mitotic division it is metabolite deficient and cannot grow without external substrate. The binucleate pollen has not exhausted its metabolites in cell division, however, and can grow until these are exhausted and it needs substrate from the style. This is the point at which inhibition occurs in the gametophytic system. Brewbaker and Majumder 1961 considered that these differences are further borne out by the in vitro germination of the two classes of pollen, binucleate will germinate on artificial medium, trinucleate shows little or no response.

Relatively little work has been carried out on the physiology of incompatibility in the Cruciferae.

In 1956, Kroh showed that if pollen of Raphanus raphanistrum was introduced into the conducting tissue of a flower of the same plant, then a normal seed set was obtained. This procedure was repeated by Tatebe 1959, who obtained the same result. Further experiments by Kroh 1966, in which she transferred individual pollen grains from self to cross stigmas and vice versa showed that pollen was irreversibly activated by a compatible stigma and reversibly inactivated after contact with a self stigma. Christ 1959 observed circular holes with smooth rims in the cuticle of Cardamine pratensis where a pollen tube had entered. These were also observed by Hobbelen 1960. Selfed pollen did not produce these holes and it was suggested that the incompatible pollen tube lacked enzymatic activity to dissolve the cuticle. Christ also suggested that the pollen tube must pierce the cuticle to obtain sufficient water for germination. Linskens 1961 agreed with Christ and investigated the cutinase enzyme further. He found (Linskens and Heinen 1962) that where the stigma has a cuticle, the pollen has a cutinase enzyme and plants without stigma cuticle produce pollen which lacks cutinase. He found high cutinase activity in B.campestris and B.nigra. In 1964, Tatebe produced further evidence that water is important for pollen germination, when he obtained increased pollen germination and a slightly increased seed set with high air humidity. Pandey 1967 stated that in Nicotiana peroxidase isozymes are involved in the expression of the S-alleles, but Nasrallah, Barber and Wallace 1969, found no segregation of peroxidase isozymes in B.oleracea.

Various gene models and hypotheses have been put forward to explain incompatibility and individual authors have altered their theories over the years. From his early work on mutation of the S-gene in *Prunus avium* and *Oenothera organensis*, Lewis 1949 concluded that the S-gene consists of at least two parts, one operating in the pollen and the other in the style with no crossing-over between the two. All the mutations which he obtained resulted only in a breakdown of incompatibility. The breakdown could be permanent or revertible, but no new S-specificities were produced as a result of mutation either spontaneous or induced by X-rays (Lewis 1951, 1954, Lewis and Crowe 1954). Pandey 1956 produced similar results with *Trifolium pratense* and *T. repens*, and in 1957 Rajan produced a breakdown in incompatibility in *Brassica* by irradiation at the tetrad stage. This gave further support to the hypothesis of sporophytic control in *Brassica*. Since Jost 1907 pointed out the similarity between the inhibition of pollen tubes through incompatibility and the immunity reaction of animals, many workers found East's 1929 hypothesis on the subject very acceptable, Sears 1937, Lewis 1942, Linskens 1954. However, Moritz 1958 assessed the hypothesis and concluded that an enzyme-antienzyme reaction is more likely than the stimulation of antibodies. Accordingly, Lewis and Crowe 1958 suggested that the specific pollen protein is an enzyme essential for pollen tube growth and that this enzyme is inactivated by a homologous anti-enzyme in the style containing the same S-allele.

In 1960 Sampson put forward his hypothesis of a threshold level of incompatibility substance produced by an S-allele, which is needed before the effect of that allele is expressed. The hypothesis involved competition for a common limited factor, equal competition resulting in independent action, unequal competition to dominance. In the same year, Lewis put forward a gene model where the S-gene consists of two cistrons. One controls the specificity of the S-allele and the other activity in pollen and style. This model was favoured as a result of his experiments with diploid pollen, and in 1963, further experiments caused him to put forward his protein dimer hypothesis. According to this, a dimer is produced in both pollen and style and in the incompatibility reaction, identical dimers combine to form a tetramer with the aid of an allosteric molecule such as glucose, a protein or one of many small molecules. The tetramer acts as a genic regulator either to induce the synthesis of an inhibitor or repress the synthesis of an auxin of pollen tube growth.

In 1962, Pandey put forward his theory of S-gene structure. This suggested that the physiology of the S-gene is controlled by four components; a growth substance, a protective substance, a primary specificity controlling interspecific incompatibility and a secondary substance controlling intraspecific incompatibility. Each component has corresponding pollen and style units. In the same year, Sampson suggested that in the Cruciferae, pollen and stigma recognise each other by areas of complementary structure. One area is the "S-allele area" and another the "species area". Thus, he hypothesised a macromolecular code with two pairs of sites, each with complementary structure to explain self incompatibility and intergeneric incompatibility.

Ascher 1966 also put forward a gene model based on the operator-regulator system of Jacob and Monod 1961. He proposed three sites of genetic activity in the pollen, a low-velocity operon controlling germination and early tube growth, a high-velocity operon responsible for tube growth through the style and a regulator to code for a specific monomer. In the style he proposed two sites of genetic activity, the two regulators each coding a specific monomer. Identical monomers in pollen tube and style results in a dimer repressor which switches off the high-velocity operon in the pollen. This model was intended to explain only gametophytic self-incompatibility and as such could not apply to the instances in B.oleracea where germination on the stigma does not occur.

In 1967, Mackenzie, Heslop-Harrison and Dickinson reported a drastic reduction in the ribosome population of pollen mother cells during mid-prophase of meiosis. Heslop-Harrison 1968 pointed out that this reduction coincides with the time of S-gene action in the gametophytic system. This would lead to direct competition for ribosome sites for the synthesis of S-protein and other proteins such as for mitosis. For this reason, pollen mitosis is delayed, as well as the attainment of the maximum level of S-protein. Some growth of the pollen tube occurs, therefore, before inhibition levels are reached. For the sporophytic system, he suggested that the S-protein was donated by the tapetum, thus avoiding competition for ribosome sites in the pollen mother cell. Pandey 1970a disagreed with this latter point, and considered that the S-protein was produced by the meiocyte itself. In 1969, Pandey put forward his theory that intraspecific incompatibility evolved from interspecific incompatibility early during the evolution of the angiosperms, through duplication of the interspecific incompatibility cistrons. Redifferentiation of the duplicate complex produced the large number of alleles controlling intraspecific incompatibility.

As previously stated, spontaneous and induced mutations in the S-gene led only to breakdown of incompatibility, Lewis 1949, 1951, 1954, Lewis and Crowe 1954, Pandey 1956, Brewbaker and Natarajan 1960, Sharma and Boyes, 1961. In 1963, Denward reported that new S-specificities arose regularly in <u>Trifolium pratense</u> during inbreeding. After a few generations, these new S-alleles became stabilised. Denward's explanation was that the action of the S-gene is strongly dependent upon the general genetic background of the cell and that specificity of the incompatibility reaction is determined by the entire genotype of the plant. Inbreeding modifies the overall genetic background and, therefore, affects the behaviour of the S locus. De Nettancourt 1969 disagreed with the explanation and agreed with the suggestion of Lundquist 1965 that the new specificities were produced by intra-cistronic crossing-over. A tentative alternative put forward by De Nettancourt was based on the theory of Edström 1968 that chromosomes can store information obtained during meiosis on all the other alleles segregating in a population. Thus a new specificity could be displayed when needed to increase the level of genetic polymorphism in the population to which the plant belongs.

Further cases of new specificities on inbreeding have been reported by De Nettancourt and Ecochard 1969 and De Nettancourt et-al 1971 in Lycopersicon peruvianum and by Pandey 1970b in Nicotiana. In 1972, Pandey put forward his theory to explain the generation of new S-specificities applying the fine and course controls of recombination (Simchen and Stamberg 1969) found to occur in fungi, to angiosperms. He suggested that the fine control system governs the generation of new S-alleles as the recessive genes of this system increase recombination. Thus, on inbreeding homozygous recessive alleles are produced which increase recombination thus giving rise to new S-alleles.

Serological methods were first applied to the investigation of incompatibility by Lewis in 1952. He raised antisera to pollen extracts of Oenothera organensis and on absorption these gave strong reactions only with common S-alleles. In 1960, Linskens raised antisera to both pollen and stigma extracts of Petunia. Since the specific antibody was identical in both tissues, it appeared that the S-substance detected was the same in both sexes. Both these workers used tube tests to detect the specific precipitates, but in 1962, Makinen and Lewis used gel plates in which up to three precipitation bands were formed. It appeared from their staining tests and heat denaturation that the antigenic substance was probably a protein. In 1967, Lewis, Burrage and Walls produced precipitation with single pollen grains showing that the substance diffuses readily from this tissue.

In 1965 came the first report of the application of the technique to B.oleracea. Nasrallah raised S-substance specific antibodies to three S-alleles and in 1967a found that the S-substance was not present in any part of the plant other than the stigma. In 1967b he followed the segregation of the specific band in the F_1 and F_2 and found that it correlated with the segregation of the S-allele as tested by pollination and seed set. The S-substance appeared to be a protein in this case also, and electrophoretic separation showed segregation of two S-proteins through the F_1 and F_2 generations. (Nasrallah, Barber and Wallace, 1969, Nasrallah, Wallace and Savo, 1972).

Various methods have recently been found to overcome the incompatibility barrier in B.oleracea. Tatebe 1968 achieved this with ether or 10% potassium hydroxide applied in very small amounts to the stigma. Nakanishii, Esashi and Hinata 1969 produced the same result with a carbon dioxide concentration of over 0.3%. The maximum penetration of tubes occurred at 4.5% carbon dioxide. High temperature was found to break the incompatibility barrier by Gonai and Hinata, 1971b. They observed that growth of the papilla cells was accelerated during the treatment and suggested that retardation of papilla-cell formation was associated with self-incompatibility. In 1971a they compared the papilla cells of self compatible and self incompatible strains and found that the relative growth rates of papilla cells were lower in the self incompatible strains. Roggen, Van Dijk and Dorsman 1972 obtained seed set after passing a direct electric potential difference of 100 volts between pollen and stigma during pollination. In the same year, Roggen and Van Dijk achieved the same result by pollinating with a brush of steel wire. This caused mutilation of the stigma surface, thus allowing tubes to grow.

Compatible and incompatible pollinations have been observed by electron microscopy and scanning electron microscopy in an attempt to elucidate the underlying mechanism. Kanno and Hinata 1969 produced electron micrographs which showed that the cuticle layer of the cell wall of the papilla disappeared at the point of contact with the pollen tube. They concluded that the final barrier to self pollination was the cellulose pectin layer. Roggen 1972 using scanning electron microscopy suggested that the wax layer on the papillae is the incompatibility barrier. Using the same technique, Ockendon 1972 did not come to the same conclusion but considers that the various manifestations of incompatibility are all different aspects of a single physiological mechanism, the diffusion of a specific substance onto the stigmatic surface. From the evidence presented, this appears to be the most likely possibility.

CHAPTER 2.

MATERIALS AND METHODS.

Antiserum production and gel tests.

The following standardisations were used by Nasrallah and adopted in this study unless otherwise stated. The stigma extract was prepared by removing the stigmas from the flowers and grinding them in a pestle and mortar with buffer in the proportion of 25 stigmas in 0.1 mls of medium. The extract was transferred to a tube and centrifuged at 10,000 r.p.m. for one minute to remove cell debris. Extracts such as these were used in the gel plates, those used for injection were of variable concentration. The stigma extracts used for absorption of serum were prepared as described, but 30 stigmas were extracted in 0.1 mls of medium. A sample of serum was absorbed in the proportion of three parts serum to one part absorbing stigma extract. The mixture was left for one hour at 21°C and overnight at 0°C. The precipitated proteins were centrifuged down and the supernatant used for gel tests.

An antiserum was raised by injecting a rabbit with stigma extract. Three different types of injection were given and are referred to as i/v, i/m and s/c. i/v is an intra-venous injection. These were given into the marginal ear vein of the rabbit. No adjuvant was used for these injections. i/m is an intra-muscular injection. These were given into the hind leg muscle in 1:1 emulsion of stigma extract and Freund's complete adjuvant, as supplied by Difco. This is an emulsion of killed mycobacteria in oil. It has the effect both of retaining the stigma proteins in the oil so that they are broken down more slowly by the rabbit's defence systems, and stimulating the antibody-producing system of the rabbit by the presence of whole bacterial cells. s/c is a sub-cutaneous injection. These were given into the fold of loose skin on the back of the neck of the animal. Here again, a 1:1 emulsion with adjuvant was given.

The blood was obtained by bleeding the rabbit from the marginal ear vein. All rabbits were bled before any injections were given and again after, and in some cases during, the injection period. Once the blood had been collected it was incubated at 37°C and allowed to clot for one hour. After this time the serum was decanted off and centrifuged to remove any remaining red cells. The serum was stored by freezing. In some cases sodium azide was added to suppress fungal and bacterial growth to give a final concentration of 0.002%.

A wider range of well spacings was used in this study than by Nasrallah; in the main, two types of gel plate were set up. The first was the dilution plate and this measured the response of the rabbit to the injections by showing the number of antibodies built up and the titre of each. Well pattern 2 (see later) was used for dilution plates as this pattern was found to give the best separation and clearest bands. The centre well was loaded with a normal stigma extract prepared exactly as the extract used to inject the rabbit except that it was of the standard concentration of 25 stigmas in 0.1 mls of buffer. The outer wells were loaded with two fold dilutions of the serum from undiluted down to a maximum of 1/2048. A dilution plate was set up for every blood sample taken and every plate was repeated. If a constant result was obtained then every band visible was given a number. The band nearest the antiserum wells was called band 1, the next band 2, and so on until the band nearest the antigen well was reached. The bands in each serum were numbered separately so that, for example, t_1 of one serum does not necessarily represent the same antigen-antibody complex as t_1 of another serum. Titre of an antibody was reported as the highest dilution of antiserum at which the antibody produced a precipitation band against the standard stigma extract concentration. The titre of each antibody was followed through successive bleeds. The change in titre of each antibody was then plotted on a graph to show the progress more clearly. The main problem with these plates was to identify a particular band from one batch of serum to another. This was done by comparing the batches of serum in one gel plate against the standard stigma extract. A reaction of identity was shown between identical antibodies in different sera by the continuity of the band between the two wells.

The other type of plate was the genotype-comparison plate. A range of well patterns was used for these plates. The centre well was loaded with absorbed or unabsorbed serum and the outer wells with stigma extracts from a variety of genotypes. A band continuous between two wells showed that the two stigma extracts contained a protein in common. Again all plates were repeated.

In all but a very few stated instances the slides used for the double diffusion tests in gels were Ilford Transparency Cover Glasses, 5 cm x 5 cm in size. Having standardised on the choice of slide and the concentration of stigma extract, there were many other variables to be considered and tested before a standard repeatable technique could be decided upon. These variables included the choice of extraction and dilution medium, the choice of gel medium, the size of the wells, the spacing of the wells, the temperature of incubation of the gels and the state of the stigma extract and absorbed sera, whether freshly-prepared or having been stored in the deep freeze.

Immunoelectrophoresis was carried out according to the methods of Grabar 1959. Details of the technique are given later. Paper chromatography was carried out according to the methods of Bate-Smith 1948. Again details are given later.

<u>Genotype testing by pollination and ultraviolet microscopy</u>.

Each plant tested by gel diffusion had previously been tested for its S-allele constitution by controlled pollination followed by ultraviolet microscopy of the pistil. This revealed whether or not the pollen tubes had germinated and grown through the stigma and style. The use of aniline blue and uv microscopy for assessing the compatibility of pollinations of <u>Brassica</u> was reported independently by duCrehu 1968 and van Hal and Verhoeven 1968. These workers had adapted the methods of Linskens 1957 and Martin 1959.

The expected S-allele constitution of most plants was known as a result of testing, during the previous two seasons, of the parents of the plants by J.R.T.Hodgkin. I tested all the plants used in this study as follows; four tests were carried out on each plant in a segregating family, a self, a cross with a tester plant containing each of the possible S-alleles and an outcross to a tester containing an S-allele not expected in the plant. The latter three tests were done in reciprocal also. The number of tests was reduced to three in the case of a plant homozygous for its S-allele, but a diallel was carried out on all plants in the family to detect possible contaminants. The self pollination was to measure the level of self incompatibility of the plant. If a plant gave an average of more than 20 tubes on selfing it was not used to provide extracts for gel tests. The crosses to the tester plants were carried out to determine the S-allele genotype of the plant. If the plant contained the same S-allele as the tester then no pollen tubes would grow. If it did not contain the S-allele, then full compatibility occurred with at least 200 tubes growing down the style. The final cross was carried out to ensure that the plant was showing full fertility and was capable of producing at least 200 pollen tubes on a compatible style and of allowing at least 200 tubes to grow down its own styles.

The tests were done in reciprocal to allow for dominance relationships in one tissue but not in the other, as well as to show the level of fertility of both pollen and stigma. For example, in a plant heterozygous for S_{45} S_{15}, S_{45} is dominant to S_{15} in the style, so no pollen tubes are produced with the pollen from an S_{45} tester plant, but tubes are produced with S_{15}-containing pollen. Thus, in a family segregating for S_{45} and S_{15}, the homozygous $S_{45}S_{45}$ plants cannot be distinguished from the heterozygous $S_{45}S_{15}$ plants except by the reciprocal cross to the S_{15} tester plant as female. Due to the lack of dominance in the pollen, the pollen from the homozygote would grow, but not the pollen from the heterozygote. Fortunately no situations were encountered where one allele was dominant to the other in both pollen and style as progeny tests must be carried out on such a segregating family.

The method of testing was to select three freshly-opened flowers for each test. Each of these was emasculated by removing the anthers with a pair of forceps sterilised in alcohol. Each stigma was brushed by an anther bearing fresh pollen from the appropriate plant. The raceme was labelled and the flowers left for 24 hours. The gynaecium of each pollinated flower was then removed and the three gynaecia from one test placed together in a labelled tube containing the following fixative: 120 parts absolute alcohol, 60 parts chloroform and 20 parts acetic acid. The gynaecia were left overnight to clear and fix and were then rehydrated by transferring to deionised water via 70% alcohol and 30% alcohol, 10 minutes being allowed in each medium. The gynaecia were then softened by incubating them for one hour at 60°C in 0.8N sodium hydroxide after which they were left in aniline blue overnight. They were then mounted in 80% glycerol and observed at a magnification of X150 through a microscope adapted for uv microscopy with an HBO 200 high pressure mercury vapour lamp. The aniline blue stains the callose in the pollen grain and tube and the callose plugs in the pollen tube. This stained callose fluoresces a green/yellow colour under uv light so that the tubes can be distinguished from the style tissue and counted.

In 1971, twelve families were tested by the methods described to determine the S-allele constitution of each plant. Two families were segregating for S_2 and S_{15}, four for S_{45} and S_{15} and six for S_2 and S_{45}. The intention was to isolate the homozygotes and use the plants homozygous for one S-allele to inject a rabbit. The plants homozygous for the other S-allele were to be used to absorb the resulting antiserum. Since all the plants in one family were derived by selfing a single parent, it was hoped that the plants would be sufficiently similar for complete absorption of all but the S-allele specific proteins. This was particularly likely as the parents themselves had been produced by inbreeding, so this was likely to be the most uniform material available.

Of the two families segregating for S_2 and S_{15}, both were found to include some partially self-compatible plants, leaving too few useful homozygous plants. Also, one of the families gave only two S_2 homozygotes and no S_{15}- homozygotes and all plants were very weak.

One of the families supposedly segregating for S_{45} and S_{15} was found in fact to be segregating for

S_2 and S_{45}. Of the remaining three families none included any S_{15} homozygotes and only one included a single $S_{45}S_{15}$ heterozygote. For some reason the S_{15} allele appears to be at a disadvantage in association with the S_{45}-allele and S_{15} homozygotes are rarely found segregating from an $S_{45}S_{15}$ family. A possible explanation is that in this material the S_{15} allele is linked with a gene conferring reduced viability on the pollen. The fact that most of the brussels sprout material at S.H.R.I. is closely related may explain why there are no families in which the S_{15} allele is not at a disadvantage in association with the S_{45} allele.

There were now seven families segregating for S_2 and S_{45} and these were the only remaining possibilities for providing homozygotes for injection and absorption. Of the seven, two included partially self-compatible individuals and one showed a tendency toward male sterility. One family produced only S_{45} homozygotes, one produced no S_2 homozygotes and one no S_{45} homozygotes. This left three suitable families of which most of the plants were heterozygotes. The few homozygous plants were singled out but by the time this work had been completed all had passed their main flowering period (flush) of the season. The plants were all very weak as they were so inbred, and many of them produced very few flowers after the first flush, some producing none at all. Since so few flowers were available, none of these plants was used for injection extracts, but some were used to prepare absorption extracts. The plants used to give vaccine were chosen on their ability to provide a large number of flowers over a relatively long period of time.

During the course of this work it was found that the S_{45} allele is dominant to the S_2-allele in the pollen. There is independent action of the two alleles in the stigma.

<u>Propogation of plant material</u>.

By pollinating a stigma with its own pollen while it is still in the bud, the self-incompatibility mechanism can be overcome and selfed seed is produced. Bud pollination was the method used to produce all seed. Not only can two plants containing the same S-allele be crossed, but the seed produced is also less likely to be contaminated with foreign pollen as the bud is so much more protected than the open flower.

The sepals and petals were prised apart on each of about twenty buds on two or three racemes per plant. If the plant was selfed, the anthers were left intact, but they were removed with forceps if the plant was crossed. The stigmas were brushed with a ripe anther from the appropriate plant. The petals and sepals were replaced as far as possible and the racemes labelled. When ripe the seed could be harvested and sown the following year.

Most <u>B.oleracea</u> varieties are biennial, so no flowers are produced the year the seed is sown. This means that the progeny from a particular plant will not produce flowers until two years after the parent plant was in flower. However, the life cycle can be reduced from two years to one by germinating the seed 60 days after pollination. <u>Brassica</u> seed is fully mature by this stage and is green in colour. Normally this green seed remains on the plant and after a further 10-20 days becomes brown and passes into a dormant phase which continues until the following spring. Seed taken from the plant 60 days after pollination will germinate to produce normal plants. If the bud pollinations are carried out in March, then the green seed can be germinated in May and the resulting plants transplanted to the field in June. They will then grow and receive the cold temperatures needed to vernalise them. They will flower in the following spring if the plants are brought into the glasshouse sufficiently early.

All plants used to provide stigmas for injection were bud pollinated and the seeds green germinated as described. This was done both to maintain the plants as similar to the parents as possible and also to cross the plants with other S-genotypes. If a specific S-antibody was raised, the segregation of the protein could be followed and compared with the results obtained by pollination and uv microscopy.

<u>B.oleracea</u> plants treated as described stop flowering and die after seed production in autumn. A method of extending the availability of flowers up to the end of the year is to vernalise the plants artificially, (Miller 1929, Stokes and Verkerk 1951, Heide 1970). Five-week-old plants will bolt and flower if subjected to a temperature of between 4 and 7°C for 6 to 9 weeks. Seed at S.H.R.I. was germinated in autumn and the plants vernalised during the summer so that the plants were flowering by autumn. These plants

flowered until the end of the year and in some cases into the beginning of the following year. These plants could be bud pollinated and green seed germinated as for any naturally-vernalised plants.

Some seed of *Brassica alboglabra* was obtained from Dr. Ockendon of the National Vegetable Research Station (N.V.R.S.) in 1972. This is a variety of *B.oleracea* despite its own species name and is fully compatible with cabbage, kale and brussels sprout. It is an annual form which does not require vernalisation, so flowers are produced within three months of sowing the seed. The plants grown from Dr. Ockendon's seed contained the S-alleles S_1, S_5, and another unknown allele. Brussels sprout and kale plants with other S-alleles were crossed with these plants in the hope that the plants grown from the seed produced would retain the annual habit and also have a wider range of S-alleles relevant to this work. The F_1 seed obtained from a cross between the *B.alboglabra* and a brussels sprout of S-allele constitution $S_{45}S_{45}$, were germinated and grown in the glasshouse at 18°C. The plants received no vernalisation, and of the 99 plants, 53 flowered and 46 did not. It is unlikely that a single dominant gene is involved. Further progeny tests, keeping the plants at a range of temperatures are needed before the mode of inheritance of the character can be determined.

B.oleracea plants can also be successfully propogated by cuttings (North 1952, 1953). This is another method of preserving useful plants.

CHAPTER 3.

TESTS CARRIED OUT WITH A VIEW TO IMPROVING THE TECHNIQUE.

The agar used for gel diffusion tests until July 1971 was 'Oxoid' Agar No. 3. This was used at a 1% concentration as was found to give the clearest firm gel in which wells could easily be cut. 0.5% agar gave too soft a gel and 1.5 and 2% gave very rubbery gels which were difficult to cut and which were cloudier than the 1% gel, making observation of faint bands more difficult. After July 1971 Oxoid Ionagar No. 2 was used. This gave a clearer gel that the 'Oxoid' No. 3 and was also used at a 1% concentration. Tests were carried out to compare the different concentrations of the agars, but no differences in banding patterns were produced.

The volume of agar per slide was also varied. Antisera 1 and 2 were used to compare the different volumes and no differences in banding pattern were observed between 8 and 12 mls of agar per slide, 10 mls per slide was decided upon as standard as it gave the best cover of the whole slide without spilling before solidifcation of the agar.

In some of the early tests it was evident that some leakage of reagents was occurring between the gel and the slide. To prevent this all slides were coated with a water-repellent silicone surface before use. 'Repelcote' supplied by Hopkin and Williams Ltd. was used. A dilute solution of bromphenol blue in phosphate/saline buffer was used to test for leakage. The dye diffused into the gel, but leakage beneath the gel occurred before this on the uncoated but not on the coated slide.

Using 10 mls of agar per 5 cm x 5 cm coated slide and well pattern 1 (see later) the centre well was of 0.2 mls capacity and each outer well was of 0.05 mls capacity.

Early in 1972 tests were carried out to compare agarose gels with agar gels. The agarose supplied by B.D.H. gave a clearer gel than agar of comparable concentration and was tested in a range of concentrations. Leakage tests with bromphenol blue showed that this was not a problem with the agarose gel, so uncoated slides were used. The agarose would not completely cover a coated slide presumably due to its having different surface tension properties from agar.

Agarose gels of 0.5, 0.75 and 1% concentration were compared with the 1% agar gel. All gave clearer gels than the agar. Antisera 6, 8 and 10 were used to compare the gels. Dilution tests showed that no differences in resolution were obtained using the different gels, but that 0.5% agarose gave the clearest gel. The S_{45}-specific band was visible in both 1% agar and 0.5% agarose gels. Photograph 1 shows the S_{45}-specific band, which is the inner band, as seen in a 1% agar-gel. Photograph 2 shows the identical plate set up with 0.5% agarose.

10 mls of agarose per slide had been used in all these tests, but this volume was not easily accomodated on the slide at the 0.5% concentration. 8 mls per slide gave less spillage before solidification of the gel.

To compare the two volumes, twofold dilutions were prepared of antiserum 10 taken 13.6.72. and plates set up. Photographs 3 and 4 show results in the 8 ml gel, and photographs 5 and 6 show results in the 10 ml gel. No difference could be seen between the two gels, so 8 mls of 0.5% agarose gel was used per slide from July 1972 onwards.

Using 8 mls of agarose per 5 cm x 5 cm uncoated slide and well pattern 1, the centre well was of 0.15 mls capacity and each outer well was of 0.0375 mls capacity. All gels, both agar and agarose contained 0.05% sodium azide to suppress fungal and bacterial growth. Plates were set up to find if the azide had any effect on the banding patterns. Sera were compared with and without azide as were the gels. No differences in banding patterns were observed using antisera 2, 4 and 6, but fungal and bacterial colonies soon appeared on the gels which did not contain azide.

The medium used by Nasrallah to extract the proteins from the stigmas was a 0.1 molar phosphate/saline buffer of pH 7.4. An easier medium to prepare was saline, 0.85% sodium chloride, at its own pH of 6.5 or adjusted to pH 7.0 with 0.1 N sodium hydroxide. This was also the medium used to prepare the dilutions of the sera. A comparison of the two media used for extraction of stigma of an S_{45}-containing brussels sprout plant against dilutions of antiserum 8 taken 2.2.72. showed very little difference between the two media. Photographs 7 and 8 show the banding pattern given by freshly-prepared saline and phosphate/saline buffer respectively in 1% agar gels. The saline gave slightly clearer results and so was used as extraction medium from the beginning of 1972 onwards. However, the phosphate/saline buffer was still used to some extent as it maintained its pH better than the saline.

Many tests were carried out varying well sizes and well spacings. Most of these have been described under the antisera involved. In general, larger wells revealed more bands, but above 7 mm diameter wells banding patterns became confused due to increased precipitation of the stronger bands masking the fainter ones. Below 3 mm diameter, wells did not contain sufficient reagents to give distinct banding patterns.

As can be seen from photograph 9 showing a comparison of well spacings, the two smaller spacings 4 mm and 6 mm gave better resolution of the five bands visible than the two greater spacings of 8 mm and 10 mm. This was done with 6 mm diameter wells and antiserum 8 taken 2.2.72. A 3" x 1½" glass microscope slide was used. It was decided that the well pattern most suited for clearest separation of the maximum number of bands was one of seven wells each of 6 mm diameter, the six outer wells each being 6 mm from the centre well.

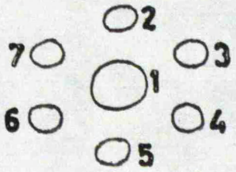
Tests on the S-protein band showed that smaller outer wells placed closer to the centre well gave maximum resolution of this band. This was the pattern described by Nasrallah 1967 and is described in the table of well patterns used in this study, (Table 1).

TABLE 1.

Table of well patterns.

Pattern 1: Seven wells. Inner well 8 mm diameter, outer wells 4 mm diameter. Outer wells 4 mm from inner well rim to rim. A template was made for this pattern. The wells were numbered as follows:

Diagram 1:



Pattern 2: Seven wells, all 6 mm in diameter, outer wells 6 mm from inner well rim to rim. A template was made for this pattern. The wells were numbered as for pattern 1.

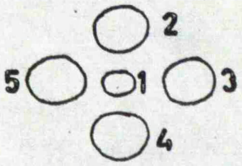
Pattern 3: Seven wells, all 7 mm in diameter, outer wells 5 mm from inner well rim to rim. Wells were numbered as for pattern 1.

Pattern 4: Seven wells, all 7 mm in diameter, outer wells 3.5 mm from inner well rim to rim. Wells numbered as for pattern 1.

Pattern 5: Seven wells, all 5 mm in diameter, outer wells 3.5 mm from inner well rim to rim. Wells were numbered as for pattern 1.

Pattern 6: Five wells. Inner well 4 mm diameter, outer wells 8 mm diameter, outer wells 4 mm from inner well rim to rim. The wells were numbered as follows:

Diagram 2:



All wells were cut with the appropriate size of cork bores until templates were made for well patterns 1 and 2.

To find the best temperature for development of the bands, identical plates were set up and left to develop at different temperatures. Constant temperatures only were used as a change in temperature is known to cause splitting of bands in some cases (Crowle, 1961). Direct comparison of a large number of temperatures could not be carried out as few incubators were available. In general, temperatures below 10°C caused very slow development of banding patterns and sometimes bands did not migrate so far as at higher temperatures but tended to precipitate on other bands. Patterns took up to a week to develop fully. Above 30°C, patterns developed rapidly, but bacterial growth was also increased. It was decided that a constant temperature between 20 and 25°C was best for development of banding patterns as results could confidently be read less than 48 hours after loading of the wells and bacterial growth was limited so as not to present a problem.

At first, no attempt was made to standardise on the humidity of the air in the petri dish in which the gel was placed. The filter paper lining the base of the petri dish was moistened with distilled water. During the course of the work, particularly with antiserum 8, it was thought that some unreliability of results may have been caused by the water content varying from gel to gel due to differences in the humidity of the environment. This would cause the gels to swell by different amounts and would affect the migration and concentration of the proteins. Early in 1972, therefore, a standard volume of 1 ml of distilled water was used to moisten the filter paper. Whenever possible freshly-prepared gels were used, but otherwise gels were stored in their petri dishes in sealed polythene containers in a constant temperature incubator set at 24°C.

Another variable which affected the banding patterns obtained in the gels was the state of the stigma extract, whether freshly-prepared or having been frozen and thawed. A considerable amount of protein was lost when stigma extract was frozen and thawed and was apparent as a yellow precipitate. However, the most convenient and least time-consuming method of preparing stigma extract was to collect 200 or more stigmas and store the extract in the deep freeze until it was needed. This was much quicker than collecting only enough stigmas for one set of plates each time a test was set up. It also meant that a serum could be tested after a plant had ceased to produce flowers.

The loss of protein after freezing and thawing was also apparent in the gel plates. Photograph 10 shows a plate set up with unabsorbed antiserum 2 taken 8.7.71. The agar gel with well pattern 1 was loaded as follows:

Well 1. As 2, 8.7.71, unabsorbed.
2. 7872, S_2S_2, fresh, b.s.
3. 8232/9, $S_{45}S_{45}$, fresh, b.s.
4. 8538, $S_{15}S_{15}$, fresh, c.
5. 7872, S_2S_2, frozen, b.s.
6. 8232/9, $S_{45}S_{45}$, frozen b.s.
7. 8538, $S_{15}S_{15}$, frozen, c.

In all cases fewer bands were visible against the extracts which had been frozen than against the freshly-prepared extracts. Also, the remaining bands were sometimes fainter. No S-protein-specific antibody was present in antiserum 2, so the effect of freezing and thawing on the S-protein cannot be seen from this plate.

Nasrallah (1967b) had stated that the S-protein was not affected by freezing. This was shown in this work also as can be seen from a comparison of photographs 11 and 12. The plates were identical except that frozen extracts were used in plate 12. The S_{45}-specific antibody was involved.

A long-term experiment was designed to test whether or not stigma extract deteriorated on storage. 2 mls of extract of S23 stigmas was prepared and this was tested against dilutions of antiserum 6 in agar gels with well pattern 2 both before and after the extract was centrifuged. The remainder of the extract was frozen and thawed out the following day so that another plate could be set up. The extract was then frozen again anf tested at monthly intervals for two months and then a year after the first plate had been set up.

Photograph 13 shows the results before the extract was centrifuged, photograph 14 shows the results after centrifuging. It can be seen that all the protein is not extracted by this method, but that some is spun down with the cell debris. After freezing and thawing (photograph 15) the middle of the three bands was not visible, the protein had been precipitated out of solution. Thereafter, the same banding pattern was obtained without change even a year after the extract was first prepared. As a result of this experiment, all stigma extracts after maceration of the stigmas were placed at 0°C for up to four hours. This was to ensure complete extraction of all protein from the cell debris, so that as little as possible was lost on centrifuging the extract. No tests of this nature have been carried out using absorbed serum containing S-protein-specific antibody, but these results indicate that stigma extract does not deteriorate very greatly on storage in the deep freeze.

Another long-term experiment was set up using antiserum 10 to compare the saline normally used as an extraction medium with a glycerol/saline medium. Heitfuss et al (1959) showed that such a medium stabilised the proteins in cabbage leaves as shown by the results of electrophoresis of the proteins on starch gels. A medium containing glycerol is also widely used in the extraction medium for pollen for allergen vaccine production. The glycerol medium used was a 50% glycerine-water solution containing 0.85% sodium chloride.

Dilutions were prepared of a sample of the serum taken 13.6.72. and tested against a freshly-prepared stigma extract in each medium. The two extracts were then stored in the deep freeze and thawed out the following day for another set of dilution plates to be set up. The glycerol medium did not freeze, but remained in solution, so no precipitation of proteins occurred as in the saline extract. Further tests were set up at monthly intervals for four months until no more of the extracts remained. Over this period no degeneration of either extract occurred except that a band was lost from the saline extract after the extract had been frozen once. This band was not lost from the glycerol extract, but the titres of the remaining bands did not change with time.

Since it had been shown that the S-protein was not lost on freezing, it was decided that the saline medium should be used as it was easier to work with than the glycerol medium.

Tests were set up with the two sera which contained definite antibodies of the S-protein to find how long the serum/stigma extract mixture needed before full absorption of the unwanted antibodies had occurred. Antisera 8 and 10 were absorbed and genotype comparison plates set up from immediately after the serum was absorbed, at 24 hour intervals up to a week after absorption. In the plates set up immediately, absorption had occurred in the gel due to rapid diffusion of the absorbing proteins from the antiserum well. This produced a cloudy precipitate in the gel around the antiserum well and masked the specific S-protein bands especially in antiserum 8 where the titre of the S-antibody was low. After the serum had been left for 24 hours at 0°C, complete absorption had taken place and no more precipitate was produced. Clear bands were formed in the gel and no improvement occurred with serum which had been left for longer periods.

The next step to be tested was whether or not the absorbed serum could be stored in the deep freeze without deterioration as could the unabsorbed serum.

Genotype comparison tests with antisera 8 and 10 showed that the specific band was still visible in absorbed serum which had been stored for up to four months in the case of antiserum 10. No precipitate occurred on thawing and there was no loss of resolution. The same was done with antiserum 12 which had been absorbed. This did not contain an S-protein specific antibody, but photographs 16 and 17 show that no change in banding pattern occurred after the absorbed serum had been stored for two weeks in the deep freeze. Photograph 16 shows the serum freshly-absorbed and 17 shows the serum after it had been frozen for two weeks.

Harrington, Fenton and Pert (1971) and Kostner and Holasek (1972) had reported that high molecular weight polymers such as polyethylene glycol (P.E.G.) increased the sensitivity of immunodiffusion reactions. P.E.G. of molecular weight about 6,000 was incorporated into agarose gels at concentrations from 0.4 to 4%. Dilution plates were set up with antiserum 6 to test whether any bands were visible to higher dilution than in the control plates of agarose. The incorporation of 0.4% P.E.G. into the gels gave a clear gel but no increased sensitivity over the control gels without P.E.G. As the concentration of P.E.G. was increased, the gel became both cloudier and softer until at 4% the gel was almost impossible to cut. The overall titre appeared to have increased by one dilution at 3.5 and 4% P.E.G. but these gels were so cloudy that could not definitely be concluded. It was concluded that P.E.G. offered no advantage in increasing the sensitivity of the gel tests.

In 1973 an attempt was made to concentrate absorbed serum. This was to reduce unreliability of results due to low titre of the S-protein antibody. Antiserum 6 taken 3.3.72. was absorbed with a mixture of T2, T4 and T6 stigmas. The absorbed serum was concentrated with the appropriate volume of 'Lyphogel'. This is a polyacrylamide gel, available from Gelman Instrument Company, which concentrates macromolecules by absorbing water and low molecular weight substances such as salt. The absorbed serum was concentrated by removal of half the volume of fluid and identical plates were set up with concentrated and unconcentrated absorbed serum. The results can be seen from photographs 18 and 19, where 19 shows the concentrated serum. The specific band was present against wells 2, 3, 6 and 7 in both cases, but the band was more distinct in plate 19. The other non-specific bands were also clearer in plate 19. The Lyphogel, therefore, increased the resolution of all the bands with no apparent loss of antibody component.

Apart from the work already described, dilution and genotype-comparison tests were carried out on all thirteen sera, some of which consisted of more than one batch, as the rabbit had been re-injected to produce further sera. During 1972 some work was done on the possibility of extracting proteins from the stigmas and pollen by diffusion rather than by grinding the tissue. This was shown by Nasrallah 1968 with single stigmas. This is described under antisera 7 and 10.

Other work which commenced in 1972 was immunoelectrophoresis, protein determination, pollen germination and paper chromatography. These are described later.

Conclusions.

From the experiments carried out it was concluded that the best gel medium for the double diffusion plates was 0.5% agarose. 8 mls was used per uncoated 5 cm x 5 cm glass slide and 0.05% sodium azide was incorporated into the gel to suppress fungal and bacterial growth. The azide did not affect the banding patterns. This combination gave the clearest gel for observing precipitation bands. Using well pattern 1, the centre well was of 0.15 mls and each outer well of 0.0375 mls capacity.

The most widely used well patterns were numbers 1 and 2. Well pattern 1 was used for the genotype comparison tests and had a central well of 8 mm diameter surrounded by six wells each of 4 mm diameter. The outer wells were 4 mm from the inner well. Using this pattern the best resolution of the S-protein-specific band was obtained. The well pattern used for the dilution plates was well pattern 2. This also had seven wells, one central and six peripheral, all of 6 mm diameter with 6 mm between each outer well and the central well. This pattern gave best separation and resolution of the maximum number of bands.

The extraction medium most used was saline, 0.85% sodium chloride, adjusted to pH 7.0 However, 0.1 molar phosphate/saline buffer of pH 7.4 was used to some extent as it retained its pH for longer than the saline.

The loaded gel was placed in a petri dish with a filter paper lining moistened with 1 ml of distilled water. They were incubated at 24°C and results were read after 48 hours.

It was found that most protein was extracted from the macerated stigma tissue if it was left to extract for up to 4 hours at 0°C before centrifuging down the cell debris. Freshly-prepared extract of 25 stigmas in 0.1 mls of buffer was often used but most tests were set up with extract which had been stored in the deep freeze. After the initial freezing and thawing, a large amount of protein was lost from the extract as a precipitate. After this little if any protein was lost from the extract on freezing and thawing for up to a year. The precipitated protein did not include the S-protein.

Other conclusions from this work were that a glycerol/saline extraction medium did not give any useful advantage over the saline medium. The S-protein was not lost on freezing and the advantage of the glycerol/saline medium was that it did not freeze, with the result that no protein precipitation took place. Absorption of the serum was complete after 24 hours and the absorbed serum could be successfully stored in the deep freeze without any loss of resolution. The incorporation of P.E.G. into the gel did not increase the sensitivity of the precipitation reaction, as had been found by other workers with different systems.

Concentration of absorbed serum using Lyphogel was found to be very successful and appeared to be a possible aid to producing a reliable S-allele diagnosis. Unfortunately it was not tested until 1973 when this work was terminating, so it was not assessed fully.

CHAPTER 4.

ANTISERA RAISED BY DR. NASRALLAH.

Dr. Nasrallah spent three months at the S.H.R.I. during the summer of 1970 and raised six antisera in six separate rabbits. Three of these he found to have S-protein-specific antibodies. Very little of these sera was left on Dr.Nasrallah's departure so only limited tests were carried out.

S-protein-specific activity was detected in one of the sera, serum 5 which was raised to a stigma extract of an S_{SD7}-homozygous brussels sprout. S_{SD7} was an S-allele detected by Dr. Johnson in the breeding material at N.V.R.S., Wellesbourne. It was called S_{SD7} until it was identified as one of Dr. Thompson's numbered alleles. S_{SD7} is of intermediate dominance. A sample of serum was absorbed 3:1 with an extract of thirty stigmas in 0.1 mls of buffer. The stigmas were collected from a range of plants all homozygous for the S_2-allele. The absorbed serum was tested against a range of genotypes homozygous for either S_2 or S_{SD7}. Agar gels and well pattern 1 were used. Photograph 20 shows that a single band was produced against all the S_{SD7}-containing genotypes, but against none of the S_2-containing genotypes.

Serum absorbed as described above was used to measure the dilution end-point of the S-protein present in the stigmas of two plants. Plant 8344/3 was heterozygous S_2 S_{SD7} and plant 8344/8 was homozygous S_{SD7} S_{SD7}. Both were the progeny of a plant which was heterozygous S_2 S_{SD7} and were produced by bud-selfing this plant. Extracts were prepared of stigmas from each of the plants and twofold dilutions down to 1/512 were made with buffer. Well pattern 1 was used and all except well 7 were loaded. This was left empty so that an accurate end-point could be read, without curvature of bands against wells 1 and 6 casting doubt upon an end-point of 1/32 against well 7. Photographs 21 and 22 show the end-point of 8344/8. The highest dilution at which a band is visible is 1/128. Photograph 23 shows a band visible to a dilution of 1/16 fainter than that in 21 and a further plate, of which there is no photograph showed a band at 1/32 dilution. The end-point of the S_{SD7} homozygous plant was 1/128. That of the S_{SD7} heterozygous plant was 1/32. This result suggests that the homozygote had more S-protein than the heterozygote. The tests were not repeated as very little serum remained and efforts were concentrated upon building up fresh sera. It would appear from this that the homozygote contains four times as much S-protein as the heterozygote. This is not conclusive, however, as extracts were prepared on numbers of stigmas rather than on weight, volume or protein content of stigmas.

The S_{SD7} allele had not at the time been identified as any of Dr. Thompson's range of S-alleles but was thought to be his S_{30}. S_{45} or S_{46}. During a visit to Cambridge, pistils were collected from Dr. Thompson's kales of these three S-genotypes and were stored in small glass tubes containing silica crystals. On reaching Dundee, the tube containing the S_{30} pistils was broken and the pistils destroyed. Extracts were made of the S_{45} and S_{46} stigmas exactly as for the usual stigma extracts. These extracts were tested along with the 8344/8 and 8344/3 against serum 5 absorbed as previously described. The plate was set up as follows:

Well 1. Serum 5 absorbed.
   2. 8344/8, S_{SD7} S_{SD7}, b.s.
   3. S_{45} S_{45} kale.
   4. 8344/3, S_{SD7} S_2, b.s.
   5. S_{46} S_{46} kale.
   6. 8344/8, S_{SD7} S_{SD7}, b.s.
   7. Empty.

The results can be seen from photograph 11. A clear reaction of identity can be seen between the band visible against well 3 and that visible against wells 2 and 4. No such band is visible against well 5.

This plate was repeated the following week exactly as above with the remainder of the extracts which had been frozen. Fresh serum was absorbed as previously. Wells 3, 5 and 6 were not completely filled. Photograph 12 shows that the bands were fainter but the band against the S_{45} kale was undoubted, both in its existence and in its continuity with the S_{SD7}-band. No band was visible against the S_{46}. It would appear from this that the S_{SD7} allele is identical with Dr.Thompson's S_{45}. The S_{SD7} allele will be called S_{45} from now on.

No S-protein-specific antibody was detected in any of the other 5 antisera raised by Dr.Nasrallah. Only limited tests could be carried out, however, as so little of each serum was left.

Conclusions.

Antiserum 5 raised by Dr. Nasrallah at S.H.R.I. contained an S_{SD7}-specific antibody. The S_{SD7}-allele was shown to be identical with the S_{45}-allele isolated by Dr. Thompson. No S-allele-specific antibodies were detected by the limited tests carried out on the other five sera. More S_{45}-protein was detected by a dilution end-point test in a plant homozygous for the S_{45}-allele than in one heterozygous for the allele. This test was not repeated, but the result indicates that a difference was present.

CHAPTER 5.

ANTISERA 1 & 2.

Rabbits 1 & 2, both Californians, were given completely different injection schedules to find which was the better for production of antiserum to the stigma proteins.

The first difference was that rabbit 1 received stigma extract which had been frozen and thawed. This was an attempt to partially purify the extract as after this treatment a yellow precipitate occurred. As shown by Nasrallah (1967b) and in this work, the S-protein was not precipitated out of solution after freezing but was left in the supernatant after centrifugation. This supernatant was used for injection. Rabbit 2 received freshly-prepared stigma extract.

The second difference was that rabbit 1 received injections over a six week period whereas rabbit 2 received only 17 days of injections. However, rabbit 1 was given 8 injections of a relatively dilute extract, as opposed to the 6 injections of more concentrated extract given to rabbit 2. The two rabbits received the same number of stigmas overall. Rabbit 1 received i/v and i/m injections only and rabbit 2 i/m and s/c injections only.

ANTISERUM 1: 7872, $S_2 S_2$, intermediate dominance, brussels sprout stigma extract after freezing.

The rabbit was bled before receiving any injections. It was then given a total of 4,500 stigmas over a period of six weeks. The first four injections were i/v given at four-day intervals. A bleed was taken ten days after the last i/v injection. The injections were then continued as i/m, further bleeds being taken a month after the first injection and thirteen days after the last. These were the only blood samples taken from this rabbit as it had tiny ear veins and was very difficult to bleed.

Dilution Tests.

Dilution tests were carried out on all samples of sera using agar gels and well pattern 2. No reaction at all was produced by the pre-injection or post-i/v sera. The reaction produced by the two later serum samples were the same. Two bands were present, both to a dilution of the antiserum of 1/8. The bands precipitated nearer to the antiserum well with increased dilution. This was because the reduced concentration of the antibodies resulted in less diffusion so equilibrium, and therefore, precipitation, between the stigma proteins and antibodies was reached closer to the antiserum well.

Genotype Comparison Tests.

Unabsorbed sera.

Plates were set up using agar gels and well pattern 1 to compare different S-genotypes against the unabsorbed undiluted sera which showed reaction in dilution tests. Serum was placed in well 1 and stigma extracts of S_2S_2 brussels sprout, S_2S_2 kale, $S_{23}S_{23}$ kale and $S_{45}S_{45}$ brussels sprout were placed in wells 2-7. The two bands present in the dilution plates were visible against all wells, no genotype differences were visible against the unabsorbed serum.

Absorbed sera.

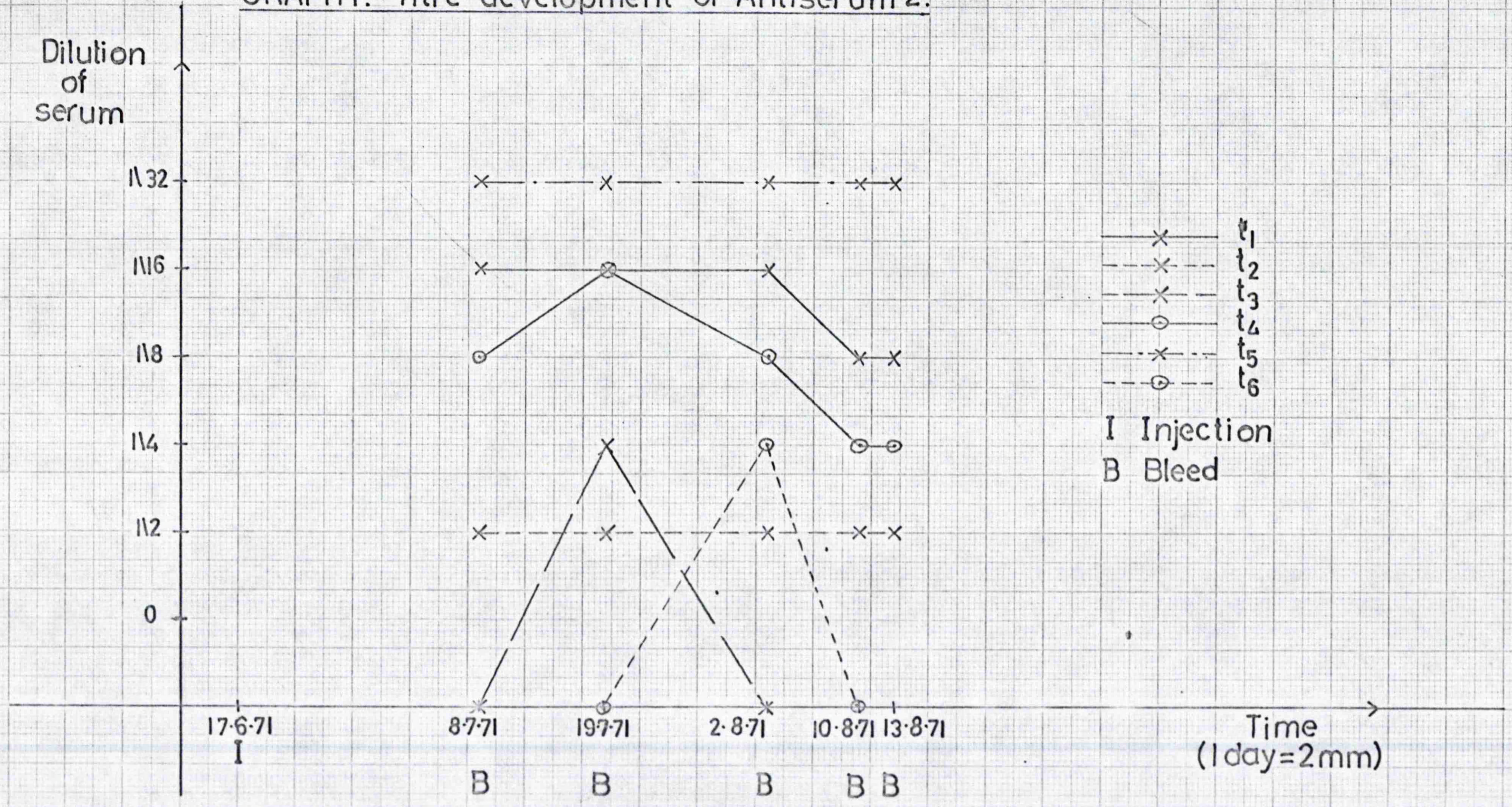
Although the same two bands were visible against all the genotypes tested, the serum was absorbed in case one or both of the bands against 7872 extract consisted of more than one component precipitating in the same position. Stigmas from five plants were used to prepare the absorbing antigen mixture. All had been tested by pollination and uv microscopy and were known to be homozygous $S_{45}S_{45}$. The two sera were absorbed in the standard proportion of 3:1, and also 4:1, 5:1 and 6:1. The absorbed sera were tested in agar gels with well pattern 1 against a range of S_2S_2 and $S_{45}S_{45}$ brussels sprout stigmas as well as an $S_{15}S_{15}$ cabbage.

In most gels, no bands at all were visible. Some faint bands could be seen against the sera absorbed 5:1 and 6:1, but these were not specific to the 7872 wells. No S_2-specific band was detected in either serum sample.

ANTISERUM 2: 7872, S_2S_2, intermediate dominance, brussels sprout stigma extract.

This rabbit received a total of 4,500 stigmas all by i/m and s/c injections over a period of 17 days. A total of six injections was given, in each case the extract was very concentrated. The rabbit was bled before receiving any injections. It was then bled three weeks after the last injection. Four further bleeds were taken the last almost two months after the last injection.

GRAPH1. Titre development of Antiserum 2.



Dilution Tests.

From graph 1 it can be seen that the maximum number of bands visible against any one serum was five. Four were present against all samples and the other two were very short-lived and were present against one sample only in each case. The date at which the serum contained the maximum titre of most antibodies was 19.7.71, which was a month after the last injection. After this, titre of two antibodies began to fall and no further titre increases occurred. The two bands visible against antiserum 1 corresponded to bands t_1 and t_5 of antiserum 2. This was shown by comparison plates set up with two antisera in one gel. The pre-injection serum showed no reaction.

Genotype Comparison Tests.

Unabsorbed sera.

Plates were set up using agar gels and well patterns 1, 2, 3, 4 and 5 to compare different S-genotypes against the unabsorbed undiluted sera taken 8.7.71. and 19.7.71. Various S-genotypes were tested including S_2S_2, $S_{45}S_{45}$, $S_{15}S_{15}$ and $S_{23}S_{23}$ kale and brussels sprout stigmas. The banding patterns produced showed many superimposed bands and no S-allele specific differences could be seen. The different well patterns made little difference to the banding patterns.

Absorbed sera.

Samples of the sera taken 8.7.71. and 19.7.71. were absorbed with various concentrations of stigma extract in various proportions and the absorbed sera tested using a range of well sizes and spacings.

All absorbing stigmas used were either $S_{45}S_{45}$, $S_{15}S_{45}$ or $S_{15}S_{15}$ as tested by pollination and uv microscopy. The concentration of stigma extract was the usual 30 stigmas in 0.1 mls buffer and also 15 stigmas in 0.1 mls buffer. The serum was absorbed in the proportion 3:1 as usual, and also 4:1, 5:1 and 6:1. In addition, each of the genotypes used in combination for absorption was also used separately in the above concentrations and volumes for absorption, to allow for any possible error in the testing of the plants. As many of these variations as possible were tested using agar gels and well patterns 1, 2, 3, 4 and 5. The absorbed sera were tested against a range of genotypes other than those used for absorption as well as a control of stigma extract of the absorbing antigens. Genotypes used for comparison were other sources of homozygous S_2S_2 apart from 7872, heterozygous S_2S_{45}, homozygous $S_{45}S_{45}$ and homozygous $S_{15}S_{15}$. All these tests could not be set up at one time, but were carried out over a period of six weeks.

In most cases no bands at all were visible. With the lower concentrations of absorbing antigens, lower volumes of absorption and larger wells, some bands were visible but none was specific to the S_2-containing genotypes.

For example, the 8.7.71. serum was absorbed 3:1 with stigmas from the following $S_{45}S_{45}$-containing plants; 8343/6, 8342/8, 8344/5, 8293/10 and 8232/1. The serum was left to absorb for three days at 0°C to allow maximum absorption to occur. The following plate was set up using agar gels and well pattern 4:

Well 1. Antiserum 2, 8.7.71, absorbed 3:1

2. 7872 frozen, S_2S_2, b.s.

3. 8232/6 fresh, " "

4. 7872 " " "

5. 8342/8 " $S_{45}S_{45}$ "

The results can be seen from photograph 24. Quite distinct bands were produced despite absorption because large wells were used. There were bands specific to 7872, but not to all S_2-containing genotypes.

Dilution of antiserum and antigen.

The main reason for varying the concentrations of the reactants was to allow for the possibility that the undiluted serum and extracts may not have been providing equilibrium conditions for the S-protein and its antibody. This was also the reason for varying the sizes and distances as well as the volumes and proportions of absorbing extract. It was possible that at the concentrations used, precipitation of the S-protein was either not occurring or was taking place too near to or actually in a well so that the band was not visible. Another reason was that precipitation may have been occurring, but the band was masked by another protein whose antibody was not completely absorbed. Altering the concentrations would prevent superimposition of bands as different relative properties and mobilities of proteins would show up.

Dilution of the antiserum in the proportions used for absorption also acted as a control to show that loss of bands on absorption was due to removal of antibodies from the serum and not merely to the dilution effect.

The first test to be tried was dilution of the antigen. The undiluted serum taken 8.7.71 was tested against a range of genotypes all diluted by half with buffer. Well patterns 1 and 5 were used and the wells loaded as follows:

Well 1. Antiserum 2 unabsorbed 8.7.71.
2. 7872 fresh S_2S_2
3. " frozen "
4. 8344/8 fresh $S_{45}S_{45}$
5. 8299/2 " $S_{45}S_2$
6. 7872 frozen S_2S_2
7. 8343/1 " $S_{45}S_{45}$

Diluted half strength with buffer

As can be seen from photographs 25 and 26, many bands were still present, more being visible in the gels with larger wells. Compared with the plates of undiluted serum against undiluted stigma extracts it could be seen that many bands were lost, but still no S_2-specific band was visible.

Dilutions of the 8.7.71. antiserum were also tried and the serum was diluted with saline in the proportions 3:1, 4:1 and 5:1. The same genotypes as described previously were used to prepare the undiluted stigma extracts and well patterns 1 and 5 were used. The results were very similar to those obtained by dilution of the stigma extracts. Fewer bands were visible than in the plates set up with undiluted serum and extracts but still no S_2-specific band was visible. These plates showed more bands than the corresponding plates set up with absorbed serum, so the lack or reduction of bands on the latter was due to absorption rather than to dilution of the serum.

Absorption and dilution tests combined.

Agar gels and well patterns 1, 2 and 4 were used. The stigma extracts were diluted to half strength with buffer and the 8.7.71. serum was absorbed 6:1 with a stigma extract from five plants, all homozygous S_{45}. The wells were loaded as follows:

Well 1. Antiserum 2, 8.7.71, absorbed 6:1
    2. 7872 frozen S_2S_2
    3. 8232/6 fresh "
    4. 7872 " "
    5. 8342/8 " $S_{45}S_{45}$.

The results for well patterns 3 and 4 can be seen from photographs 27 and 28 respectively. The bands obtained with well pattern 1 were extremely faint, but showed the same pattern as those on 3 and 4. There was very little difference between these two except that 3 was fainter than 4. Once again, 7872-specific bands were visible but not S_2-specific bands.

Conclusions.

The conclusions which could be drawn from this comparison were rather limited. Few bleeds were taken from rabbit 1, and no S_2-specific antibody was found in either serum despite the wide range of varied conditions used in the tests on antiserum 2. There is no doubt, however, that more antibodies of higher titre were present in antiserum 2. There are four possible reasons for this. The first is the difference between the state of the extract used for injection. It has been shown that a considerable amount of protein is lost on freezing and thawing of the stigma extract. The difference between the two sera, therefore, may be the protein lost on freezing. This appears to include both whole proteins and the concentration of others. However, gel tests involving fresh and frozen extracts have not shown such dramatic loss of protein, although they cannot show the antigenic state of the proteins. In other words, the freezing and thawing process may have changed the proteins so that they react in a gel test but do not produce an efficient antibody response in the rabbit.

The second possibility is the difference in the injection schedule. Both rabbits received the same number of stigmas overall, but rabbit 1 received a long schedule of dilute injection extracts and rabbit 2 the opposite. It may be that a few injections of concentrated extract over a short period of time is an advantage.

The third possible reason for the observed differences is that rabbit 1 was bled once during the injection schedule and once two weeks after the last injection whereas rabbit 2 was not bled until 3 weeks after the last injection. It is possible that the titre and number of bands do not reach the maxima until three weeks after the cessation of injections.

The fourth reason for the observed differences in the antisera is the possible different reactions of the two rabbits to the proteins of the stigma extract.

This is a factor which must always be taken into consideration where antiserum production is concerned. Two rabbits does not give a valid comparison, but availability of plant material and time limited this comparison to two animals.

CHAPTER 6.

ANTISERA 3 & 4.

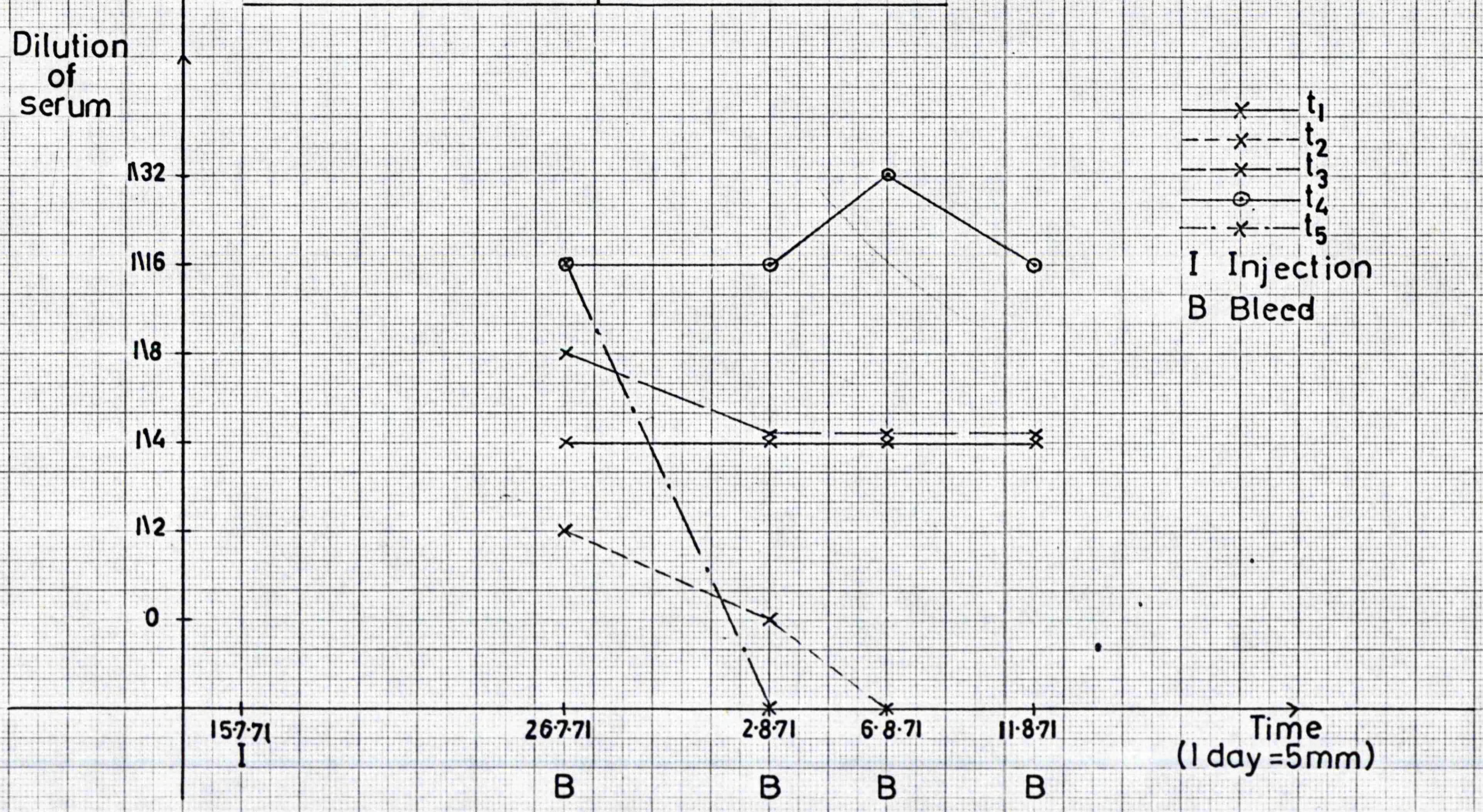
A comparison was to be made here between the injection of fresh and frozen extract as for the 7872. In this case the genotype was 8538, an S_{15} homozygous cabbage. The S_{15}- allele is low in the dominance series. The intention was to give identical injections to both rabbits so that a more direct comparison could be made than for the 7872, where the injection schedules differed also.

Each rabbit was to be given a course of i/v injections followed about a month later by i/m and s/c injections. This was the method used to produce antisera to plant viruses. The course of i/v injections was intended to sensitise the animal to the proteins so that when i/m and s/c injections were administered a rapid increase in the titre was produced in response to the proteins. Rabbits 3 and 4 were both Californians.

ANTISERUM 3: 8538, $S_{15}S_{15}$, low dominance, cabbage stigma extract after freezing.

After the pre-injection bleed this rabbit was given three i/v injections at 3-day intervals. A total of 1,500 stigmas was administered via these injections. Three weeks later a course of five i/m and s/c injections was commenced. 2,500 stigmas were given over a period of two weeks. The rabbit was bled eleven days after last injection and three subsequent bleeds were taken 18, 22 and 27 days after the last injection.

GRAPH 2. Titre development of Antiserum 3.



Dilution Tests.

No reaction was produced by the pre-injection serum. From graph 2 it can be seen that a maximum of five bands was visible against the 26.7.71. serum. After this date the titre of all but one bands either fell or, in one case, remained the same. By 6.8, 22 days after the last injection, two bands had been lost.

All the following tests were carried out on 26.7.71, as from the graph, this appeared to be the most promising batch.

Genotype Comparison Tests.

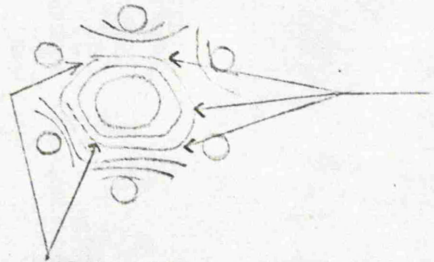
Unabsorbed serum.

The serum was tested using agar gels and well pattern 1 against the following genotypes:

Well 1. Antiserum 3, 26.7.71, unabsorbed.
2. 8538, $S_{15}S_{15}$, cabbage.
3. " " "
4. 7328/10, $S_{15}S_{45}$, b.s.
5. 8538, $S_{15}S_{15}$, c.
6. S16, $S_{16}S_{16}$, k.
7. 6167/2, S_2S_2, c.

The results can be seen from photograph 29. Clear bands were produced against all genotypes and it appeared that one band may have shown continuity between the 8538 in wells 2, 3 and 5 and the 7328/10 in well 4. Both of these genotypes contained the S_{15}-allele.

Diagram 3:



Continuity of band between genotypes

Possible discontinuity

It also appeared possible, however, that this band may have been continued against well 6. The serum was absorbed to clarify the banding pattern.

Absorbed serum.

Agar gels and well pattern 1 again were used. The serum was absorbed with a mixture of genotypes including an S_2S_2 cabbage, 6167/2, an $S_{45}S_{45}$ brussels sprout, 8232/3, an $S_{45}S_{45}$ cabbage, 8452 and an $S_{23}S_{23}$ kale, S23. The serum was absorbed 3:1, 4:1, 5:1 and 6:1. The wells were loaded as for the plates with unabsorbed serum, except that well 3 contained stigma extract from an $S_{15}S_{15}$ kale. Photograph 30 shows the results obtained with the serum absorbed 4:1, and all other plates gave the same result. One faint band only was visible against well 3 and the 7328/10 in well 4 and the band was also visible against all other wells. No S_{15}-specific band was visible.

Dilution of antiserum and further absorption.

Tests were carried out with diluted serum as for antiserum 2, and for the same reasons.. On the previous plates, more bands were visible against the kale and cabbage than against the brussels sprout genotypes. To try to eliminate these and so clarify the pattern, more cabbage and kale stigmas relative to brussels sprout stigmas were used to absorb the antiserum. The proportions used for absorption were 5:1 and 10:1 The genotypes used for absorption were as used previously except that 7872 was used rather than 8232/3. This was in an attempt to eliminate some of the outer bands produced against 8538, as these were visible when

8538 was tested against antiserum 2 raised to 7872 (see photograph 10). These proteins must, therefore, have been present in the extract injected. The plates were set up using the same genotypes as described previously.

The serum absorbed 5:1 gave results as seen on plate 30 from the previous tests. The serum absorbed 10:1 gave results as seen in photograph 31. Less absorption was produced but still no S_{15}-specific band was visible. Dilution of the serum gave results as shown on photograph 32 where the serum was diluted 5:1. The pattern was little different from that obtained with unabsorbed serum. Even when the serum was absorbed in a proportion as low as 10:1, considerable loss of antibody protein occurred.

Dilution of antigen and varied well patterns.

Plates were set up to test absorbed and unabsorbed serum against dilutions of the stigma extracts. These were diluted to half strength with buffer and absorption was 3:1 with the genotypes used in the antiserum dilution tests. A more concentrated extract of 7328/10 of 50 stigmas in 0.1 mls of buffer was prepared as this plant was heterozygous and so may have contained only half the concentration of S_{15}-protein as the homozygous 8538. Agar gels and well patterns 1, 2, 3 and 5 were used with the same genotypes as previously to compare absorbed and unabsorbed serum and diluted and undiluted stigma extracts in as many combinations as possible.

The increased concentration of 7328/10 stigma extract produced two extra bands, but neither of these was S_{15}-specific. Dilution of the extracts produced little change in the pattern obtained with unabsorbed serum, but fewer bands were visible against absorbed serum. The larger wells gave better separation of bands in all cases except well pattern 3. No S_{15}-specific band was visible in any plate.

ANTISERUM 4: 8538, $S_{15}S_{15}$, low dominance, cabbage stigma extract.

This rabbit was bled, and received 1,500 stigmas by three i/v injections at three-day intervals as rabbit 3. However, rabbit 4 received no more injections as it was returned to the source from which it was purchased with a severe and uncontrollable attack of body mites.
No antiserum was raised.

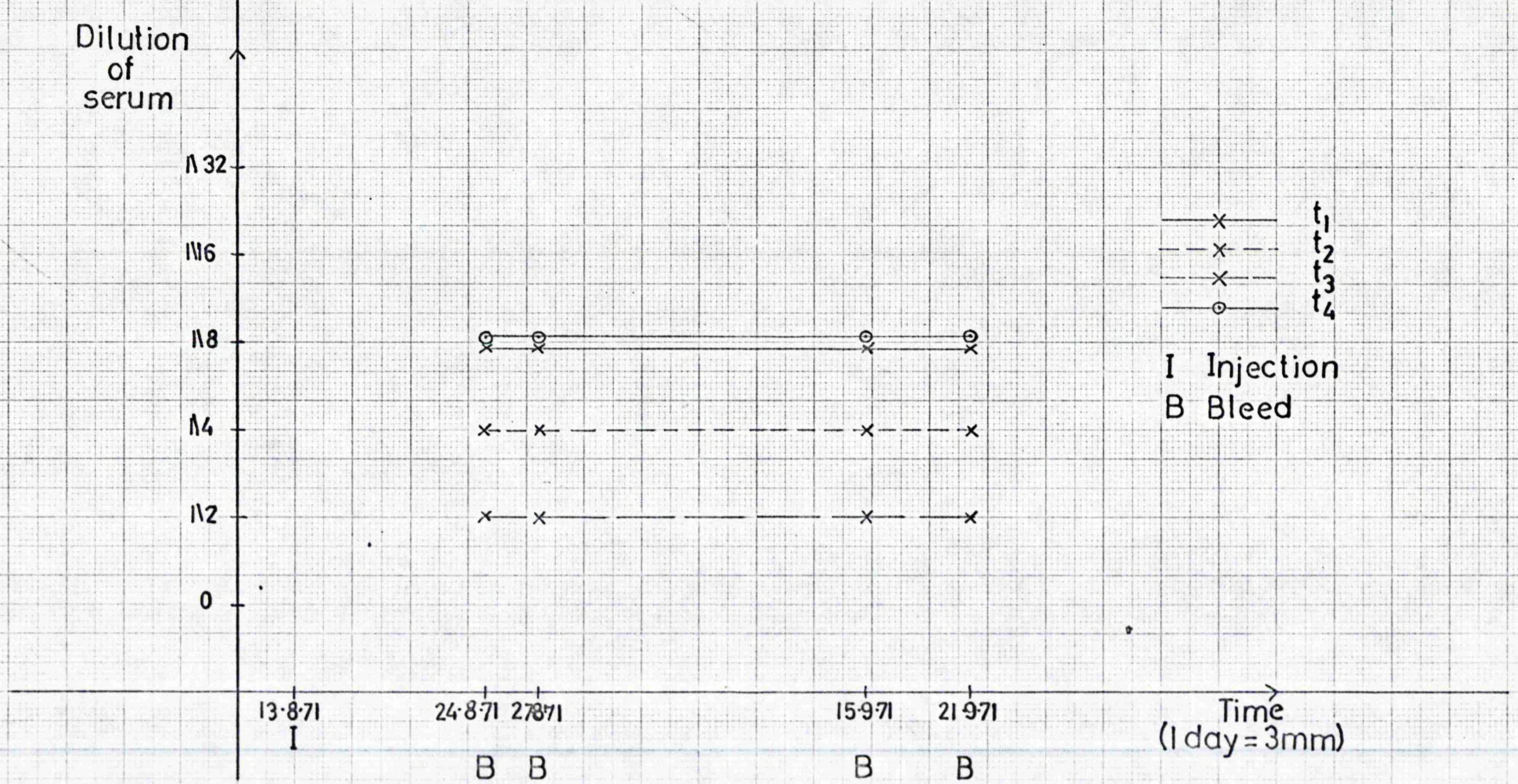
Conclusions.

No comparison could be made between the fresh and frozen stigma extracts as no antiserum was raised in rabbit 4.

As with antiserum 2, no S-protein-specific band was found in any plates despite the range of conditions tested. A difference was observed between the titre development of antisera 2 and 3. Antiserum 2 was at its higher overall titre and complexity a month after the last injection, antiserum 3 reached this situation only 11 days after the last injection. However, rabbit 2 was not bled until 21 days after the last injection so the two cannot really be compared.

In both sera, one antibody underwent a rise in titre about a month after the last injection when the titre of all other antibodies was falling.

GRAPH 3. Titre development of Antiserum 5.



CHAPTER 7.

ANTISERA 5 AND 6.

These two antisera were also raised in the hope of effecting a comparison between fresh and frozen extract for injection. Here again, however, the health of the rabbits affected the results.

Both rabbits were to receive stigma extract from S23 kale, homozygous for the S_{23}-allele which is high in the dominance series. The same injection schedule of i/m and s/c injections was to be given to each Californian rabbit.

ANTISERUM 5: S23, $S_{23}S_{23}$, high dominance, kale stigma extract after freezing.

The rabbit was bled previous to receiving any injections. It received 4,500 stigmas by seven i/m and s/c injections over the period of a month. Toward the end of the course of injections it was noticed that the rabbit had contracted a disease known as "snuffles" caused by Pasturella septica. The course of injections was completed but very little blood was taken from the rabbit. As a result very few tests were carried out on the serum and none was repeated. Bleeds were taken 11, 14, 33 and 39 days after the last injection.

Dilution tests.

No reaction was produced by the pre-injection serum. From graph 3 it can be seen that four bands were visible against all sera and that the titre of each antibody remained the same.

Genotype Comparison Tests.

Unabsorbed serum.

Agar gels and well patterns 1, 3, 4 and 5 were used to compare unabsorbed samples of all sera against a range of kale and brussels sprout genotypes. No differences in banding pattern were visible between any genotypes.

Absorbed serum.

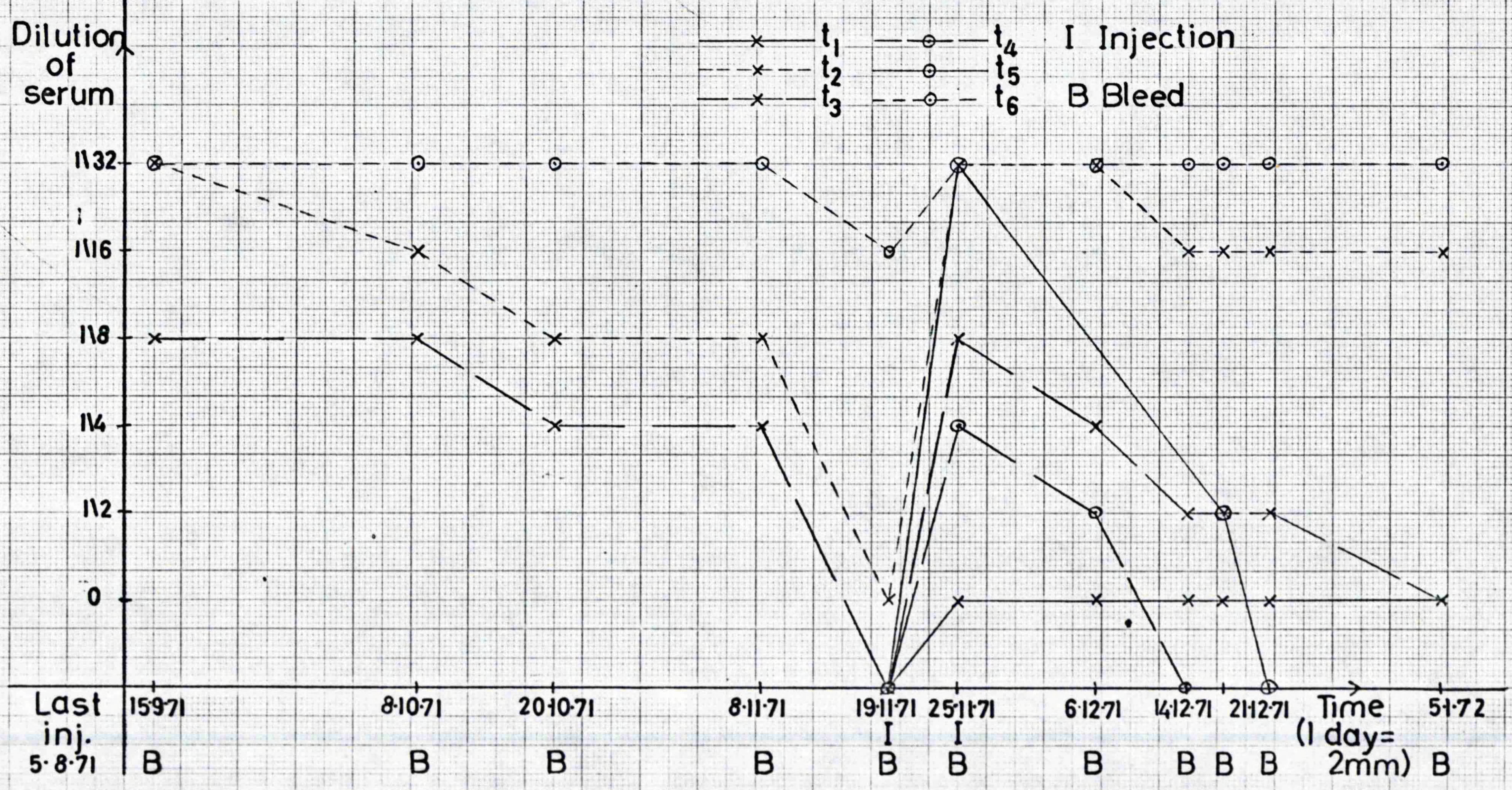
Absorption was carried out on the serum taken 24.8.71, 11 days after the last injection. Five different S-genotypes were used for absorption, three kale, one brussels sprout and one cabbage. The S-alleles involved were S_{16}, S_5, S_{17}, S_2 and S_{45}. Agar gels and well patterns 1, 3, 4 and 5 were used and the wells loaded as follows:

Well 1. Antiserum 5, 24.8.71, absorbed 3:1
2. S23, $S_{23}S_{23}$, k.
3. " " "
4. 58/17, $S_{27}S_{27}$, k/b.s.
5. S16, $S_{16}S_{16}$, k.
6. S23, $S_{23}S_{23}$, "
7. S299/2, S_2S_{45}, b.s.

With well pattern 1, no bands at all were visible. With all other well patterns, a single continuous band was visible against all genotypes. This can be seen on photograph 33 where well pattern 3 was used. There was no S_{23}-specific band.

Absorption was also carried out on the other sera using S16 kale stigmas only and well pattern 3. Results were as in the previous plates. Again there

GRAPH 4. Titre development of Antiserum 6 before and after first booster.



was no S_{23}-specific band.

ANTISERUM 6: S23, $S_{23}S_{23}$, high dominance, kale stigma extract.

This rabbit was to be given the same injection schedule as rabbit 5. After receiving 3,000 stigmas rabbit 6 developed a staphylococcal abcess on the back of its neck. This appeared to be due to the s/c introduction of vaccine and so injection of the stigma extract was ceased while the rabbit was injected with penicillin. The rabbit continued to be very weak and so was left to recover. A bleed was taken six weeks after the last injection followed by three more bleeds, 9, 11 and 12 weeks after the last injection.

When the rabbit had recovered, thirteen weeks after the last injection, it was given two more i/m injections each of 300 stigmas and separated by six days. Bleeds were taken before each injection and were continued for a further five weeks.

Dilution tests.

The pre-injection serum gave no reaction. From graph 4 it can be seen that despite the six week gap between the last injection and the first bleed, two of the three bands had a titre of 1/32. By the last bleed before the continuation of injections, only two bands remained, and the titre of both was lower than in the first bleed.

Six days after the first continuation injection, the titre of all three antibodies had increased and three new bands had appeared.

This increase in titre was not maintained, however, and the titre of all three bands had fallen by 6.12.71, despite the second injection given 25.11.71. By 5.1, two of the new bands had disappeared and the titre of the other four appeared to be relatively stable. Since an increase in titre and complexity of the serum occurred after continuation of injections, these were termed 'booster injections'. This was a manifestation of the anamnestic reaction which was recognised by von Pirquet in 1911. He found that when anaphylactic hypersensitivity was once established, it gradually waned without further contact with the antigen, whose reintroduction caused the hypersensitive state to reappear in a much shorter time than was first required.

Genotype Comparison Tests.

Unabsorbed serum.

These tests were carried out on the sera taken six and eleven weeks after the last of the first series of injections. The following plates were set up with agar gels and well pattern 3.

Well 1. Antiserum 6, unabsorbed.
2. 8299/9, S_2S_2, b.s.
3. A4 , $S_{15}S_{15}$, k.
4. S23 , $S_{23}S_{23}$, "
5. 8293/10, $S_{45}S_{45}$, b.s.
6. S16 , $S_{16}S_{16}$, k.
7. 58/17, $S_{27}S_{27}$, k/b.s.

As can be seen from photographs 34 and 35, the reaction to the 15.9.71. serum (34) was much stronger than that to the 20.10.71 serum (35). In both gels there was an outer band present against the S23 extract in well 4 which was not present against any other genotype. This band was also one of the first to develop.

A similar test was carried out on the serum taken during the second course of two injections. This serum was collected 6 days after the first booster injection and dilution tests had shown that the serum was far more complex than that taken either 15.9. or 20.10.71. These tests were carried out in November, so there were very few flowers to work with. This plate was not duplicated, therefore, and some wells were not filled. Agar gels and well pattern 3 were used and the wells loaded as follows:

Well 1. Antiserum 6, 25.11.71, unabsorbed.
2. Mixed kale stigmas, S_{15}, S_{16} and S_{27}.
3. S23, $S_{23}S_{23}$, k.
4. 8344/1, S_2S_{45}, b.s. and 7872, S_2S_2, b.s.

From photograph 36 it can be seen that there were two bands visible against the S23 in well 3 which were not visible against either of the other two wells.

Likewise with the serum taken 6.12.71, eleven days after the second booster, a plate was set up when sufficient stigmas could be collected. The gel and pattern were as before and the wells loaded as follows:

Well 1. Antiserum 6, 6.12.71, unabsorbed.
2. S23, $S_{23}S_{23}$, k.
3. A4, $S_{15}S_{15}$, "
4. 58/17, $S_{27}S_{27}$, k/b.s.
5. S16, $S_{16}S_{16}$, k.

6. 8538, $S_{15}S_{15}$, c.

7. 8299/7, S_2S_{45}, b.s.

From photograph 37 it can be seen that there was an extremely strong band against the S23 in well 2 which was not present in either the A4 in well 3 or the 8299/7 in well 7. There was, however, a fainter outer band present against wells 4, 5 and 6 but it could not be seen whether or not this was continuous with the band against the S23.

A plate was set up using the 6.12.71 serum to test whether this outer band was continuous with the S23 band and also to compare another source of the S_{23}-allele with the S23 kale. Since this work was carried out late in the year there were no plants in flower at Mylnefield which contained the S_{23}-allele other than the S23 kales. Dr. Ockendon from Wellesbourne had a brussels sprout plant of the genotype $S_{23}S_{26}$ in flower and he sent 30 gynaecia of this plant through the post in a tube with silica crystals. The stigmas were excised as normally and extracted in 0.13 mls of buffer. A slightly generous volume was used to counteract the desiccation of the stigmas during transit. The extract was used along with other stigma extracts to set up the following plate:

Well 1. Antiserum 6, 6.12.71, unabsorbed.

2. S23, $S_{23}S_{23}$, k.

3. 58/17, $S_{27}S_{27}$, k/b.s.

4. 8299/7, S_2S_{45}, b.s.

5. 70381/9, $S_{23}S_{26}$, b.s.

6. S23, $S_{23}S_{23}$, k.

As can be seen from photograph 38, the outer band present against the S23 wells was continuous with a band in the same position against the 7038/9 extract. An outer band was also visible against the 58/17 in well 3. This was rather faint and continuity was uncertain.

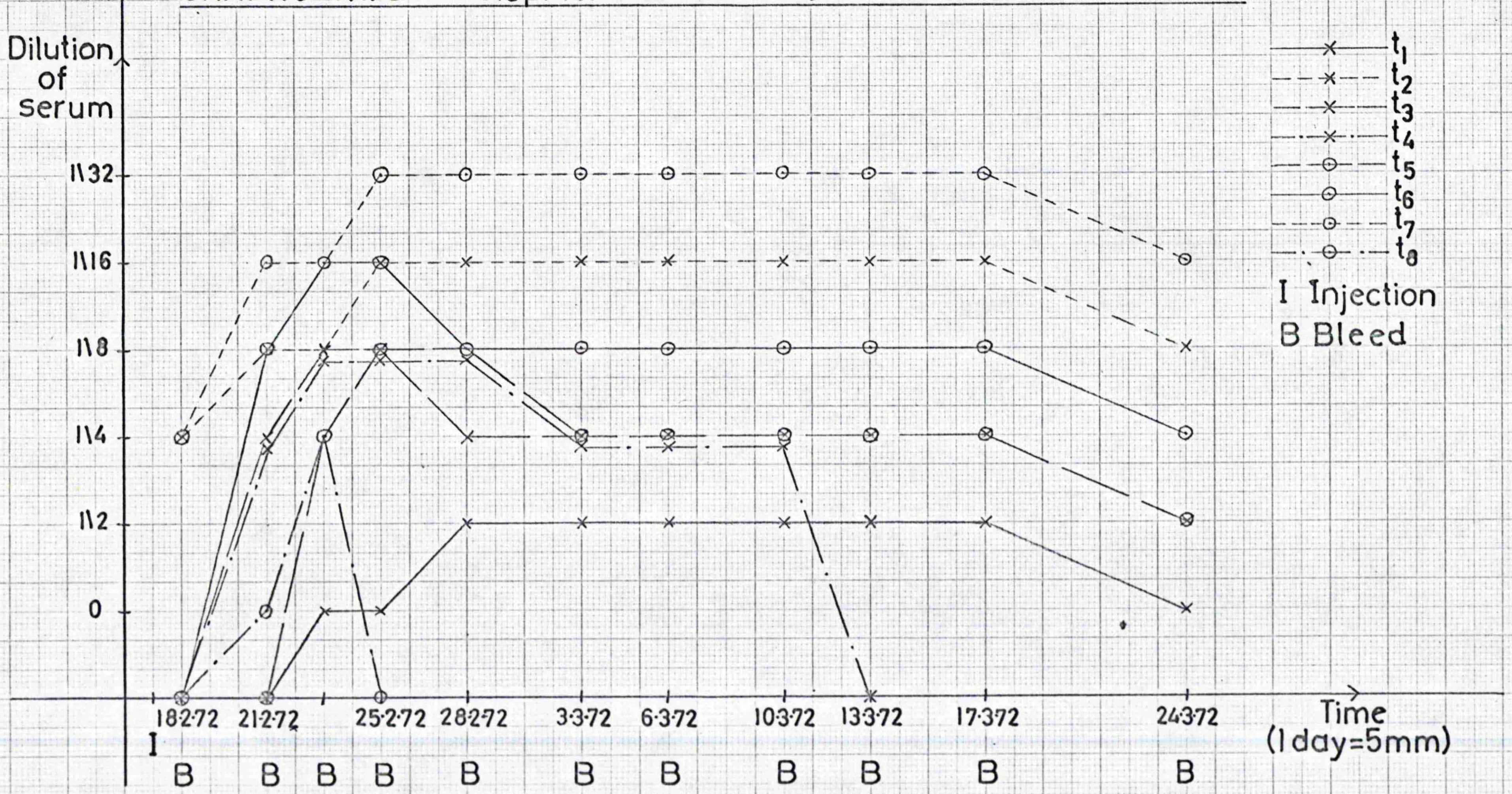
These tests were repeated in 1972, but the outer band was never detected again. Two of the S23 kales were disposed of at the end of 1971 and the outer band was not found against extracts from the four remaining plants, or the S23 plants flowering in April 1972. The latter plants were grown from seed obtained from Dr. Thompson as were the plants flowering in 1971. It would appear that this band was associated with one or both of these plants but this could not be proved. No further material was obtained from Dr. Ockendon and the band was not found against any other genotypes tested. The plates were repeated in November and December 1972 in case the protein was built up toward the end of the year, but no such band was present against any serum.(See later photographs 45-50).

Absorbed serum.

The serum taken 15.9.71, 6 weeks after the last of the first course of injections, was the only sample to be absorbed in 1971 due to lack of flowering material. Two absorbing extracts were used, S16 kale and 58/17 kale/brussels sprout. Absorption was 3:1 and 3:2. Agar gels and well pattern 3 were used and the wells loaded as for the unabsorbed serum.

3:1 absorption produced the results seen in photograph 39. All the outer bands were removed leaving

GRAPH 5. Titre development of Antiserum 6 after second booster.



only an inner band common to all the genotypes tested. The 3:2 absorption produced the same result except that the band was fainter. No plate showed the presence of the outer band visible in photograph 34. This pwotein, therefore, must have been present in the S16 and 58/17 extracts used for absorption. It was not S_{23}-specific.

1972

Booster injection given to rabbit 6.

Since such a dramatic response was obtained to the two injections given to this rabbit 19.11 and 25.11.71, it was decided to give the rabbit a booster of 300 stigmas by i/m and s/c injections. A bleed was taken the following day and further bleeds were taken at intervals of 2, 3 or 4 days for a month. This was to follow the rise and fall of titre as closely as possible.

Dilution Tests.

From graph 5 it can be seen that the effect of the injections was not apparent on the following day, 18.2.72, but that the titre and complexity had started to rise by 21.2. The serum was at its most complex on 23.2, six days after the injections when eight bands were visible. Two of these were not produced in response to the previous booster injections, when the 25.11 bleed was also taken six days after the first injection. The serum had the highest titre of most antibodies on 25.2, eight days after the injections. The titres of most antibodies were maintained for longer in response to these boosters than to the previous ones.

Genotype Comparison Tests.

Tests were carried out on some of these sera in 1972. Further tests were also carried out on the previous batches of sera produced by this rabbit. These had not been satisfactorily completed in 1971 due to lack of flowering plant material.

The S23 kale seed had been obtained from Dr. Thompson of the P.B.I. He very kindly supplied seed of other kales as closely related to the S23 kales as possible, but possessing different S-alleles. This seed was germinated at S.H.R.I. and the plants came into flower in September, 1972. All plants were tested by pollination and uv microscopy against the S23 plants. Their S-alleles could not be checked further than this as no appropriate tester plants were available. However, Dr. Thompson had ascertained that the kales were of S-genotypes S_8S_8, $S_{25}S_{25}$ and $S_{17}S_{17}$. More seed of $S_{23}S_{23}$ plants was also provided.

These plants were used for absorption and testing of some sera. Samples taken 25.11.71, 6.12.71, 14-21.12.71, 21.2.72, 23.2.72, 25.2.72 and 3.3.72 were absorbed with kale stigmas of $S_{25}S_{25}$ and S_8S_8 genotypes. The absorbed and unabsorbed sera were tested using agarose gels and well pattern 1. The 25.11.71, 6.12,71 and 23.2.71 sera were tested as follows:

Well 1. Antiserum 6, absorbed.
- 2. S23, $S_{23}S_{23}$, k.
- 3. 7471, $S_{23}S_{45}$, b.s.
- 4. T2 + T4, k - used for absorption.

5. T3, $S_{25}S_{25}$, k.

6. T6, $S_{17}S_{17}$, "

7. T7, $S_{23}S_{23}$, "

The 14-21.12.71, 21.2.72 and 25.2.72 sera were tested as for the other sera except that well 3 contained S23 stigma extract as no further 7471 extract was available. The 3.3.72 serum was tested as for the other sera except that well 3 contained T7 and well 6 contained S23 stigma extract.

Photographs 40, 41, 42, 43, 44 and 18 show the results obtained with absorbed 25.11.71, 6.12.71, 23.2.72, 14-21.12.71, 25.2.72 and 3.3.72 sera respectively. In plates 40, 41 and 42 it can be seen that although absorption was not quite complete, a single continuous band was visible against genotypes S23 and T7, both of which contained the S_{23}-allele. However, this band was not visible against the 7471 in well 3 and this had been shown by pollination and uv microscopy to contain the S_{23}-allele. The band was again visible in plates 43 and 44 against wells 2, 3 and 7, and in plate 18 against wells 2, 3, 6 and 7, although it was very diffuse and close to the antiserum well in 44. No band at all was visible in the 21.2.72 serum.

Dr. Thompson also provided a family tree of the kales involved. The S23 and the T7 were very closely related, but were also very close to the T6, $S_{17}S_{17}$ plants. The T2, T3 and T4 were also related but not so closely. If this band represented a protein in the S23 and T7 which was not absorbed by the T2 and T4, then it would be expected to be present in the T6 also as this was so closely related. However, the fact that the band was not visible against the 7471 in well 3 cast doubt upon the result.

It could also be seen from these plates that the titre of the serum had a considerable effect on the banding patterns produced. The clearest bands were produced with the serum samples taken 6.12.71 and 14-21.12.71. From graphs 4 and 5 it can be seen that these two sera had a relatively low titre of all antibodies compared with the 25.11.71, 23.2.72 and 25.2.72 sera. The least distinct band was produced by the 25.2.72 serum which had the highest titre of the most antibodies of all the sera tested. The explanation of this effect is that at high antibody titre, there is a situation of antibody excess producing an ill-defined band. According to Oudin 1952, if antibody is in excess, the band edge facing this reactant will remain stationary and only that facing the antigen will move, broadening the existing band which appears very diffuse. It was for this reason that the previous sera, in particular 2 and 3 were tested using a range of well sizes and spacings and different antigen and antibody concentrations. This was in the hope that if a specific S-antibody was present then one of these variations would give optimal conditions for precipitation.

These differences produced by titre were also apparent in the plates using unabsorbed serum. Photographs 45, 46, 47, 48, 49 and 50 show the results obtained with unabsorbed 25.11.71, 6.12.71, 21.2.72, 23.2.72, 14-21.12.71 and 25.2.72 sera respectively. For example the reaction produced with the 25.11.71 serum was greater than that with the 6.12.71 serum. It can also be seen that no S_{23}-specific outer bands were visible, as in the tests on unabsorbed serum carried out a year previously.

In 1973 it was found that the KK1 plants, which were produced as a result of a cross between S23 and 224 made in 1972, also possessed the specific protein. However, the band appeared to be fainter than in the S23 and T7. This showed that the specific band was inherited in the F_1 generation. All the plants used had been shown by pollination and uv microscopy to possess the S_{23}-allele and also the S_{16}-allele from the 224 parent. The fact that the band was fainter in the KK1, which was heterozygous for the S_{23}-allele, may indicate that the heterozygote has less S-protein than the corresponding homozygote. An indication that this may be the case was obtained with the S_{45}-allele. However, dilution tests are needed before this can be concluded.

Also in 1973, a survey was made of all the S_{23}-containing kales at S.H.R.I. Some were obtained direct from Dr. Thompson, and others from Dr. Thompson via J.R.T.Hodgkin. All plants tested possessed the specific protein.

Immunoelectrophoresis.

From September 1972 onward, samples of antiserum 6 were tested by immunoelectrophoresis after the methods of Graber 1959. This is a method whereby proteins are subjected to an electric current. This separates the proteins on their size and charge. The proteins were separated in an agar gel through which buffer carried the current. After separation of the proteins they were allowed to react with the antiserum as in the double diffusion tests.

The apparatus was supplied by the Shandon Scientific Co. The agar was a specially purified low conductivity agar made by Oxoid for immunodiffusion work. It was made up in quarter-strength 0.1 M Oxoid Barbitone Acetate Buffer. The buffer used in the tanks was 0.1 M Oxoid Barbitone Acetate Buffer pH 8.6. Eight 3" x 1" microscope slides were used per run each with three wells of 1 mm diameter separated by two 1 mm wide troughs at a distance of 3 mm from each well. The well pattern was cut with a template supplied with the apparatus, and the wells could be offset slightly to right or left of centre relative to the flow of current.

The stigma extract used for immunoelectrophoresis was a more concentrated extract of 100 stigmas in 0.15 mls saline. The extracts were run for 1½ hours at a constant current of 25 mA provided by a P.A.G.E. Quickfit power pack. This gave a voltage of 120 volts. After the run, serum was added to the trough and the bands allowed to develop for 24 hours at 24°C.

A run was set up to compare serum samples taken 25.11.71, 14-21.1271, 23.2.72 and 3.3.72. All wells were offset toward the anode to allow better separation of the cathode-migrating proteins as Nasrallah 1967b stated that the S-protein migrated toward the cathode. The upper well was loaded with S23, the middle well with a mixture of T2, T3, T4 and T6 and the lower well with T7.

All sera gave very similar banding patterns. All gave at least seven bands against the S23 extract and up to two more bands were sometimes present but were not specific to any sera. Photographs 51 and 52 show the patterns produced against the 14-21.12.71 and 25.11.71 sera respectively. The S23 and T mixture extracts gave very similar patterns, but the T7 was slightly different in the cathode-migrating proteins. It had a protein band in a slightly different position from any protein in either of the other two extracts and lacked a protein band common to the other two. The only difference observed between the S_{23}-containing genotypes and the others in the middle well was in 52 where the second to the last protein from the anode-end of the gel was present against S23 and T7 but not against the T mixture. It was not visible, however, on the other gel set up with the 25.11.71 serum.

Immunoelectrophoresis, using more concentrated stigma extract showed more bands than were visible in the double diffusion plates. This may be because some bands in double diffusion plates consisted of more than one component or because the proteins were not sufficiently concentrated for the bands to be visible. Immunoelectrophoresis should give better results than double diffusion tests as the area for the separation of the protein is so much greater. Provided antigen and antiserum are in equilibrium, all proteins to which antibodies have been raised should be apparent as bands.

The 25.11.71 serum produced a much more compact pattern than the other sera, in the cathode-migrating proteins. This may have indicated the higher titre of the antibodies which were diffusing more rapidly and widely than in the other sera. However, this was not noticed with the 23.2.72 serum which was also of high titre.

A further run was set up to compare the freshly-prepared extracts as used previously with the extract which had been stored for 7 days in the deep freeze, thawed, and centrifuged to remove the precipitated proteins. The results can be seen on photograph 53 using 14-21.12.71 serum. The anode-migrating proteins were all lost on freezing except for two very faint traces. These were changed in character after freezing and could not be seen to correspond with any particular band in the fresh extract. Tests have shown that the S-protein is not lost on freezing, and so is unlikely to be an anode-migrating protein. The cathode-migrating proteins remained unchanged on freezing.

Since no specific band had been detected by immunoelectrophoresis and unabsorbed serum, it was decided to use absorbed serum. Fewer antibodies would thus be detected so that the specific band, if present, would be more easily recognised. In 1973 runs were set up using absorbed serum taken 14-21.12.71 and 3.3.72. The sera had been absorbed 3:1 with a mixture of T2, T4 and T6 and all batches showed the band specific to S23 and T7 in the double diffusion gel plates. In most cases no bands at all were visible after immunoelectrophoresis of the stigma extracts and reaction with absorbed serum. Occasional faint bands were present, but none were specific to any genotype. The band which was so distinct in the double diffusion plates was not visible at all after immunoelectrophoresis. It appeared that the conditions in the run were not suitable for precipitation of this antigen-antibody complex.

The factors most likely to be at fault were the well sizes and spacings and the stigma extract concentration. The well sizes and spacings were those provided by the template supplied with the apparatus. Nasrallah 1967b had used larger wells and wider troughs. Some runs, therefore, were set up using 3" x 1½" glass slides with two wells only per slide each 7 mm in diameter. The central trough was 5 mm wide and was 6 mm away from each well. Concentrated stigma extract of 100 stigmas in 0.1 mls of buffer was used by Nasrallah. Extract of the same concentration was used in this study and also a dilute extract of the concentration used for double diffusion plates, 25 stigmas in 0.1 mls of buffer. With the larger well sizes and concentrated extracts, two bands were visible against all genotypes. With the dilute extracts, only one of the bands was visible, again it was not genotype-specific. Thus, fewer bands were visible with the larger wells and troughs and no specific bands were detected.

During the course of this work it was found that the concentrated stigma extracts deteriorated rapidly on storage. Within three months almost all protein as detected by immunoelectrophoresis was lost from solution by precipitation. This is in strong contrast with the more dilute stigma extracts where no protein as detected by double diffusion plates was lost over the period of a year. No visible precipitate appeared in these extracts over this period as occurred in the concentrated extracts.

No genotype-specific band was visible in any gel after immunoelectrophoresis.

Conclusions.

No comparison could be drawn between antisera 5 and 6 as the health of the rabbits interfered with both injections and bleeds. The response of the rabbits to the injections was probably affected also. Antiserum 5 gave a very poor response.

Antiserum 6 was the first where the buildup of titre was followed by regular and frequent bleeds. Response to the first course of injections cannot be assessed as the rabbit was not bled until six weeks after the last injection. Reponse to the booster-injections was rapid and dramatic. The maximum response occurred between two and ten days after the booster, a further injection apparently having little effect. After this the titre of most bands began to fall, but this happened more slowly after the second booster given in 1972. In addition, the serum became more complex after the second booster. Whether or not this is advantageous is not known as the results showing an S$_{23}$-specific antibody are not conclusive. It would appear, however, that the S-protein is not one of these two extra bands.

It was concluded that the outer band visible against the S23 extracts and unabsorbed sera in 1971 was not S$_{23}$-specific. The antibody was removed by absorption and was not detected in any of the S23 stigma extracts collected in 1972.

The serum taken 15.9.71 and absorbed in 1971 showed no S$_{23}$-specific antibody. The sera taken between 6.12.71 and 3.3.72 all showed a band specific to S23 and T7 when tested in 1972, with the exception of the

21.2.72. serum. Since this band was not visible against the 7471 stigma extract it cannot definitely be taken as being specific to the S_{23}-allele. However, the 7471 plants were very inbred and weak and this may have affected the protein concentrations.

Immunoelectrophoretic results showed no protein differences between the S_{23}-containing and the T mixture extracts using absorbed or unabsorbed serum. Even if the band was not S_{23}-specific, it should have been visible as a difference after immunoelectrophoresis. It would appear that either immunoelectrophoresis was not revealing all the proteins or that the band specific to S23 and T7 in the double diffusion plates was not, in fact, specific.

The KK1 plants, which were the heterozygous F_1 progeny of an S23 plant, shared the specific protein with the S23 parent and with all other S_{23}-containing kales. The band produced against the KK1 extract was fainter than that against all extracts of S_{23}-homozygotes. Dilution tests were not carried out on the heterozygote and homozygote, but the result may support that obtained with the S_{45}-allele, that a plant heterozygous for an S-allele may possess less S-protein than the corresponding homozygote.

From graphs 4 and 5 it can be seen that the only antibody present in all sera between 6.12.71 and 25.2.72 except 21.2.72 was t_1. The position of the band in the dilution and genotype comparison plates does not correspond, but it is possible that its migration may have been altered by the absorption and different well pattern. Identical bands are represented by the same pattern on graphs 4 and 5.

CHAPTER 8.

ANTISERA 7, 8 and 9.

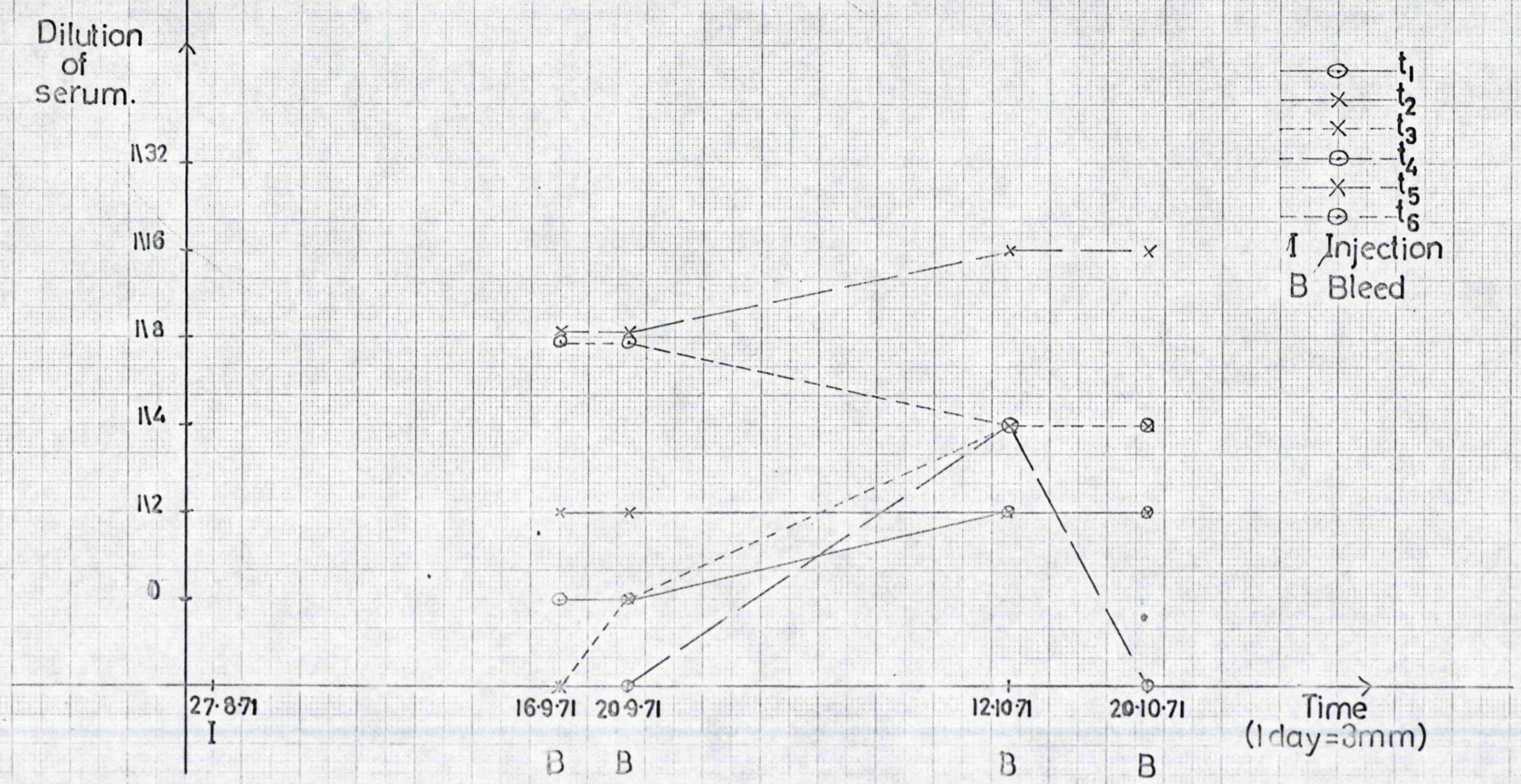
ANTISERUM 7: S23, $S_{23}S_{23}$, high dominance, kale pollen extract.

This Californian rabbit was injected with a concentrated extract of whole pollen. The first two injections were of an extract of whole anthers, but this was thought to be unnecessary when an antiserum to pollen only was required. All extractions were made in saline. A known number of flowers in excess of 500 were collected and placed in a seed sieve of mesh size 710 microns. The flowers were shaken twice and the pollen collected. The pollen was extracted in 0.1 mls saline to every 100 flowers. The mixture was ground in a pestle and mortar, pipetted into a tube and centrifuged for 5 minutes at 8,000 r.p.m. The supernatant was used for injection.

After the pre-injection bleed, the rabbit was given seven injections. The first two were anther extracts and were not standardised. The rest were pollen extracts standardised as above. The rabbit received pollen from a total of 6,000 flowers by i/m and s/c injections. It was first bled three weeks after the last injection and subsequent bleeds were taken 4, 6 and 7 weeks after the last injection.

Although the pollen extracts were standardised to the pollen from 100 flowers in 0.1 mls saline, this was far from satisfactory. The yield of pollen from 100 flowers varies considerably depending on the atmospheric conditions, the genotype of the plant and the age of both

GRAPH 6. Titre development of Antiserum 7.



plant and flowers. For example, the first flowers of a flush have less pollen than those produced later during the same flush, and far less pollen is produced under damp conditions than on a dry day. The pollen from a 100 flowers can weigh anything from zero to 0.001 gms. However, number of flowers was easier and quicker to record than weight of pollen. The flowers chosen to give pollen were all from plants in full flower and, as far as possible, the flowers were collected on the day the anthers dehisced.

Dilution Tests.

The pre-injection serum gave no reaction. From graph 6 it can be seen that a maximum of six bands was visible 6 weeks after the last injection. This was also the serum with the highest titre of most antibodies.

Comparison of Pollen Antisera with Stigma Antisera and Extracts.

The serum taken 3 weeks after the last injection was tested against pollen extract dilute and concentrated and against stigma extract. The concentrated pollen extract was the same as that used for injection. The dilute pollen extract was the pollen from 100 flowers extracted in 0.5 mls saline. The stigma extract was the usual 25 in 0.1 mls buffer. The following plate was set up with agar gels and well pattern 2:

Well 1. Antiserum 7, 16.9.71, unabsorbed.
2. S23 pollen extract dilute.
3. " " " concentrated.
4. " stigma "

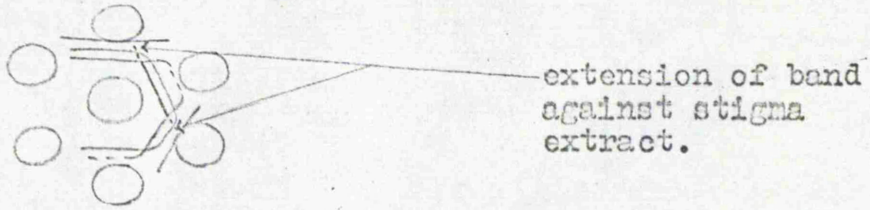
The results can be seen from photograph 54. Four bands were visible against the diluted pollen extract. Only a bright band very close to the antiserum well was present against the concentrated pollen extract as the very high concentration of all the proteins present had caused equivalence in or very near to the antiserum well. This was not suitable for gel plates and the dilute extract only was used in all further gel plates. Two bands were present against the stigma extract. These may have been proteins common to both pollen and style tissue or they may have been due to traces of pollen in the stigma extract. It was impossible to collect stigmas totally free of pollen and no attempts were made to remove the pollen adhering to the stigmas, although visible lumps of pollen were removed from the mortar while the stigmas were being excised.

The pollen antisera were also compared with some S23 stigma antisera. Agar gels and well pattern 3 were used and the following plates set up:

Well 1. Antiserum 7, 16.9.71. or antiserum 6, 15.9.71.
2. S23 pollen extract.
3. " stigma "
4. " pollen "
5. " stigma "

Photograph 55 shows the plate with pollen antiserum, 56 with stigma antiserum. As would be expected, the reaction of pollen extract against pollen antiserum was greater than that against stigma antiserum. In 55, two bands were shared by both pollen and stigma extract, one was specific to the pollen and one to the stigma. The latter was visible as a faint extension beyond the pollen bands. The banding pattern is represented diagramically as follows:

Diagram 4.



The extension of the band against the stigma extract suggests incomplete identity between the proteins in the stigma and pollen. The protein must have been present in the pollen extract or the antibody would not have been produced. However, the concentration in the two tissues may have been so different that precipitation in the same area of gel may not have been possible. Alternatively, the proteins may have existed in different forms in the two tissues so that their mobilities were changed, but their precipitation potentials were the same. For example, one may have been a polymer of the other.

In 56, there was one band present against the pollen extract and two against the stigma extract. The band against the pollen extract was probably due to contamination with pollen of the stigmas used for injection. The reaction of the stigma extracts against the two antisera was similar in both cases, indicating that these bands were produced by proteins common to both pollen and stigma. It appeared that at least two proteins were shared by pollen and stigma.

Tests were also set up with stigmas from unopened buds to see if any of the common bands were due to pollen contamination of the stigma extracts used in the tests. Other pollen and stigma antisera were compared using agarose gels and well patterns 1 and 2. The following plate was set up:

Well 1. Serum.

2. S23 pollen extract.

3. " stigma " open flowers.

4. " " " buds.

5. " pollen.

Photograph 57 shows the results with well pattern 1 and antiserum 7 taken 20.9.71. Three bands were common to both stigma and pollen extract and many extensions were produced, for reasons as already described. There was no difference in reaction between buds and open flowers, except that more precipitation was produced by the bud proteins. The bands were identical, however, showing that none of the bands visible against the stigma extract was attributable to pollen contamination. These three bands, therefore, must have represented proteins common to both pollen and stigma tissue. The outer band against the pollen extracts, which corresponded to the outer band on plates 55 and 56, showed some continuity with the stigma extracts using this well pattern. The result with well pattern 2 was similar but not so clear.

Photograph 58 shows the pattern produced with antiserum 6 taken 14-21.12.71. The sharp outer band against the pollen extract was visible and was continuous as a much fainter and more diffuse band against the stigma extract. This band was extremely sharp and well-defined even against the stigma antiserum. No bands produced by stigma proteins have been as sharp as this band and the pollen proteins in general produced clearer bands than the stigma proteins. All well patterns gave good definition of this protein-antibody system, altering the conditions, therefore, did not affect it adversely. It would appear that the nature of the pollen proteins is responsible for their clarity.

Antiserum 7 taken 20.10.71 gave a reaction very similar to that seen in photograph 57.

Plates were set up to test whether the sharpness of the pollen band was associated with its titre. Antiserum 7 taken 20.9.71 was used and pollen extract was diluted with saline. Agarose gels and well patterns 1 and 2 were used. The following plate was set up:

Well 1. Antiserum 7. 20.9.71.
2. S23 pollen extract 0
3. " " " 1/2
4. " " " 1/4
5. " " " 1/8
6. " " " 1/16
7. " stigma extract.

An end-point was not reached using well pattern 2 (photograph 59) but with well pattern 1 (photograph 60) an end-point was reached at 1/8. The dilution pattern of this band was similar to that of many stigma protein bands, so that sharpness was not associated with titre.

The plate was also set up with dilutions of stigma extract. Here the titre of the band was 1/4 with both well patterns. The band became even more diffuse with dilution.

Genotype Comparison Tests.

Absorbed and unabsorbed sera.

As with the stigma extract antisera, these tests were carried out to find if any S-genotype-specific proteins could be detected. The pollen antisera were absorbed with the extract of pollen from 50 flowers of S16 kale, $S_{16}S_{16}$ in 0.25 mls saline in the proportion of three parts of antiserum to one part absorbing extract. The serum was left to absorb as for the stigma extract sera. Agar gels and well patterns 1 and 3 were used and the sera taken 16.9.71 and 20.9.71 were tested absorbed and unabsorbed in the following plate:

Well 1. Serum.
2. S23 pollen extract, $S_{23}S_{23}$, k.
3. 7471 " " , $S_{23}S_{45}$, b.s.
4. S23 " " , $S_{23}S_{23}$, k.
5. S23 stigma extract, $S_{23}S_{23}$, "
6. S16 " " , $S_{16}S_{16}$, "
7. 8299/10 stigma extract, $S_{45}S_{45}$, b.s.

Photograph 61 shows the banding pattern produced with unabsorbed serum taken 16.9.71. Five bands were visible against both kale pollen extracts. Four were visible against 7471 pollen extract. Two bands were visible against the S23 stigma extract which showed no identity with any of the pollen bands. Neither of these bands was visible against the 8299/10 stigma extract. The reaction with well pattern 1 and the 20.9.71 serum with both well patterns was similar to this. No S_{23}-protein band was visible.

On absorption, a single continuous band was left against all pollen genotypes. This band was faint and was visible with both well sizes and sera. Photograph 62 shows the 16.9.71 serum and well pattern 3. A single band was present against the S23 stigma well. Again this showed no identity with the pollen band. No S_{23}-specific band was left after absorption.

Extraction of Proteins from Non-macerated Pollen.

A preliminary attempt was made to extract the proteins by diffusion rather than by grinding the grains. The medium used was that used by Augustin 1959 to extract allergens from grass pollen. It was called Coca's fluid and consisted of 0.5% sodium chloride, 0.275% sodium bicarbonate and 0.4% phenol. The pH of the medium was 7.8, as opposed to the 6.5 of the saline used for maceration; 0.15 gm pollen was extracted in 1.5 mls medium for 24 hours. This was the proportions used by workers with pollen allergens to give an extract containing 100,000 Noon units per ml. This was an arbitrary unit which did not take into account actual protein concentrations of extracts. Only the fluid was used to load the gel plates, no pollen tissue was included. The approximate equivalents in Noon units/ml were calculated for the macerates, but the two were not directly comparable as in the macerates, the cells had been broken and ground. Agar gels and well patterns 1 and 2 were used to compare the two in the following plate:

Well 1. Antiserum 7, 20.9.71, unabsorbed.
2. S23 pollen macerate - 200,000 Noon units/ml.
3. " " extract 100,000 " " "
4. " " macerate 1/2 100,000 " " "
5. " " extract " " " "

The results can be seen from photographs 63 and 64. Only two very faint bands were visible against the extract. This result was not expected as many workers have stated that protein diffusion from pollen is both rapid and of high concentration, Stanley and Linskens (1965), Mäkinen and Brewbaker (1967), Knox (1971). The most likely reason for the lack of detectable protein is the pH, as Stanley and Search (1971), showed that pH had a marked effect on the diffusion of proteins from pollen. No further work was carried out on the pollen, but work on extraction of proteins from S16 kale stigmas as described under antiserum 10 was taken a step further.

Conclusions.

The pollen antiserum showed a different titre pattern from any of the stigma extract antisera. The titre of three antibodies increased 6 weeks after the last injection and one new band appeared. The titre to pollen proteins was maintained longer than to stigma proteins.

The pollen proteins were also capable of producing much sharper bands than the stigma proteins. This was true against stigma antisera as well as pollen antisera, even when the protein was shared by pollen and stigma. This was not attributable to titre, and so must be due to the nature of the proteins.

At least three bands were shared by pollen and stigma tissue. One band was consistently visible between stigma extract and pollen antiserum, but not between pollen extract and pollen antiserum. This protein must be present in the pollen but in a form which cannot precipitate in the same part of the gel. This could be due to a difference in concentration or to a difference in the nature of the protein in the two tissues. This shows one of the main limitations of gel diffusion tests. The gel is incapable of acccommodating all the antigen-antibody systems in the small area of gel between the wells.

Pollen proteins differ from stigma proteins in two ways. Firstly their antibodies are maintained at relatively high titres for longer periods. Secondly they produce sharper bands in gel plates. Pollen proteins are more antigenic than stigma proteins.

ANTISERUM 8: $S_{45}S_{45}$, intermediate dominance, brussels sprout stigma extract.

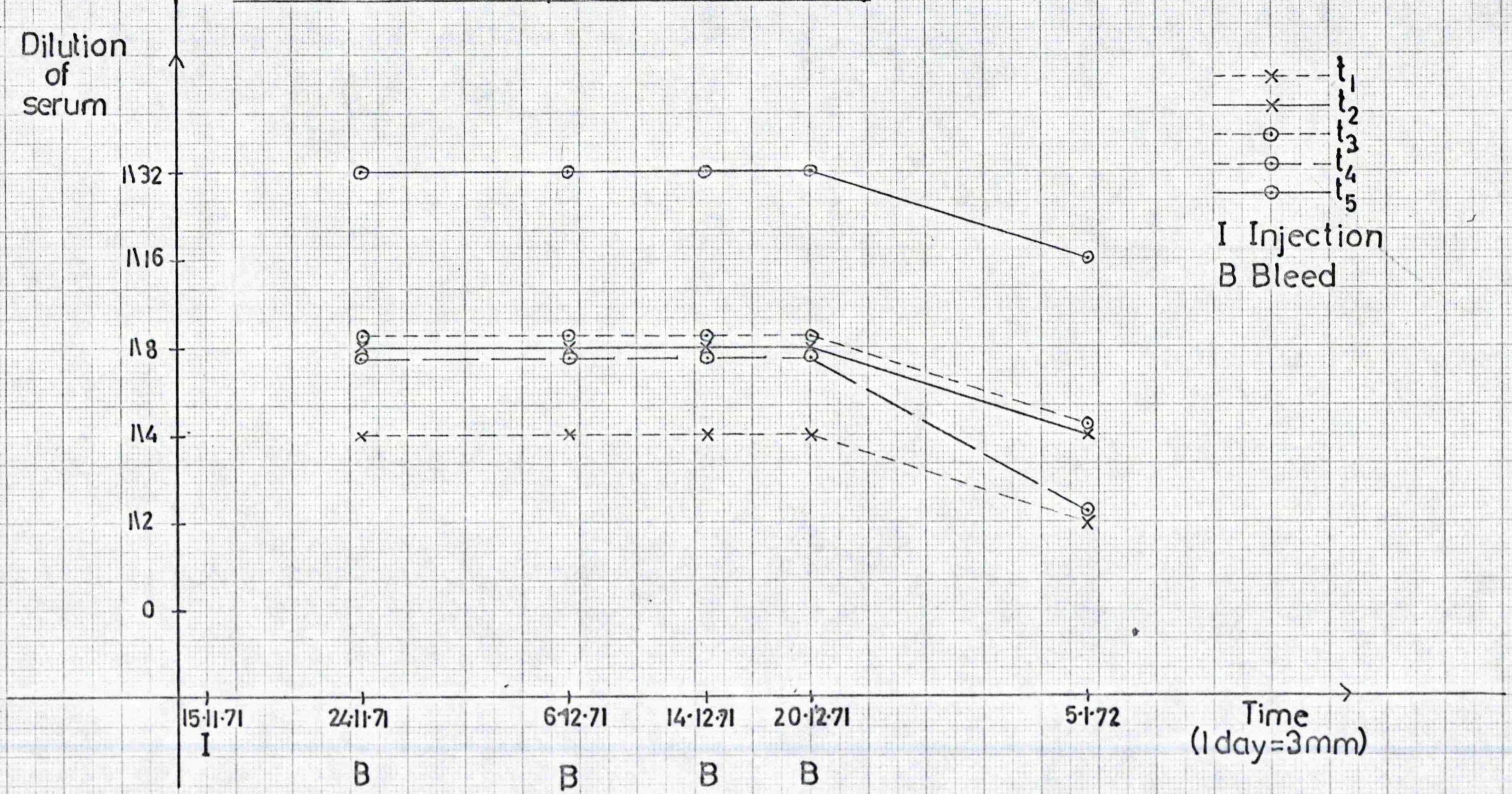
A mixture of plant lines was used to prepare the vaccine as the rabbit was injected in September, October and November. At this time of year no one line was producing sufficient flowers to inject the rabbit, so selected plants from the following lines were used: 8239, 205, 239 and 150. All the plants used had been tested by pollination and uv microscopy and shown to be homozygous for the S_{45}-allele.

After a pre-injection bleed, the rabbit was given two i/v injections separated by two days. Bleeds were taken 10 and 14 days after the second i/v injection. The rabbit then received a course of ten i/m and s/c injections at 3-day intervals until the end of the course when scarcity of flowers restricted injections to every 10 days. The doses varied from 0.25 to 1.0 mls of vaccine excluding adjuvant and the number of stigmas varied from 200 to 800. Some of the stigma extracts were more dilute than those which had previously been administered to other rabbits. The rabbit received a total of 4,500 stigmas. Bleeds were taken while the rabbit was still receiving injections, but this was toward the end of the course when the injections were reduced to one every 10 days. Two bleeds were taken before the end of the course and three were taken after the course had ended, 8, 14 and 30 days after the last injection.

Dilution Tests.

The serum taken before any injections were given

GRAPH 7. Titre development of Antiserum 8.



and the sera taken after the i/v injections gave no reaction in dilution plates.

From graph 7 it can be seen that no change in titre of any of the five bands occurred between the first four bleeds. The injection given on 6.12.71 maintained the titre of all antibodies for a further 2 weeks.

Genotype Comparison Tests.

Unabsorbed serum.

The unabsorbed serum taken 24.11.71 was tested using agar gels and well pattern 3 against a range of genotypes including $S_{45}S_{45}$, $S_{45}S_2$ and S_2S_2 brussels sprout and $S_{23}S_{23}$ kale. No S_{45}-specific band was visible.

Absorbed serum.

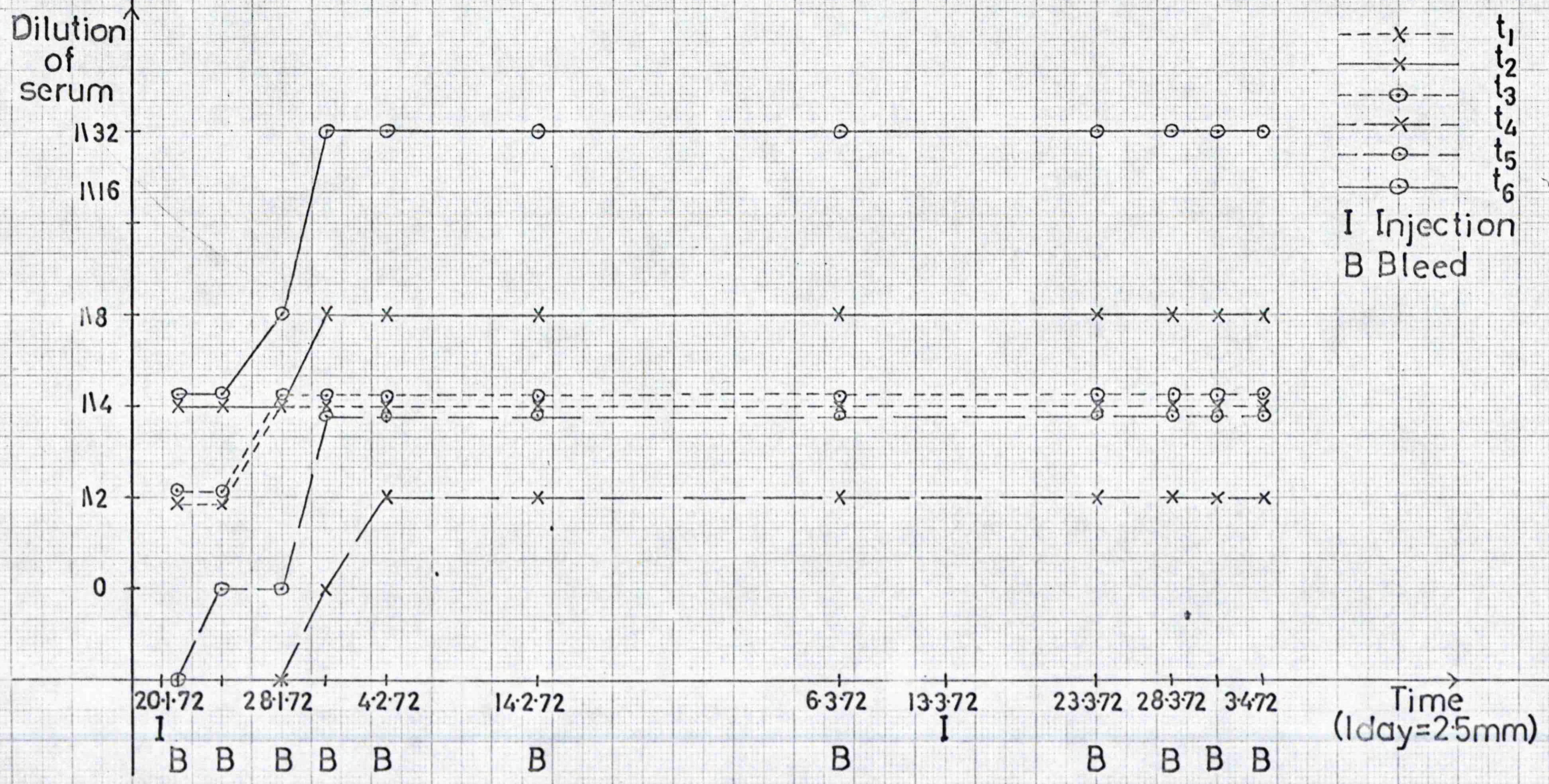
The serum taken 24.11.71 was absorbed with stigma extract from a brussels sprout line of S_5S_5 S-allele constitution. The absorbed serum was tested using agar gels and well pattern 3 against the genotypes used to test the unabsorbed serum, with the addition of the S_2S_5 brussels sprout. A few faint bands were visible in the resulting plate, but none of these was S_{45}-specific.

1972

Booster injections given to rabbit 8.

Since the booster injections given to rabbit 6 produced such a dramatic response, rabbit 8 was also given boosters and regular bleeds. The first booster was given on 20.1.72 and contained 300 stigmas in 0.4 mls saline. One i/m injection only was given as the second injection given to rabbit 6 appeared to have no effect.

GRAPH 8. Titre development of Antiserum 8 after first and second booster.



A bleed was taken the following day and further bleeds were taken for 6 weeks. On 13.3.72, a second booster was given of 200 stigmas in 0.6 mls saline by i/m injection. Bleeds were taken 10, 15, 18 and 21 days after the injection.

Dilution Plates.

From graph 8 it can be seen that increase in titre and complexity occurred between 4 and 15 days after the booster injection. This was a slower buildup than for antiserum 6. Two new bands appeared which were not visible against the 21.1.72 serum, but only one of these had not been detected previously. The titre of all six antibodies remained constant for at least a month. After the second booster injection, no bleeds were taken for 10 days. This serum showed no change in titre. The second booster injection maintained the titre of all antibodies for at least another 3 weeks. Identical bands are represented by the same pattern on graphs 7 and 8.

Genotype Comparison Tests.

Unabsorbed serum.

The sera taken 3.1.72 and 4.2.72 were tested unabsorbed using well patterns 1 and 2 against a range of stigma extracts. Only wells 1-5 were filled. The result can be seen from photograph 65, no genotype-specific differences were visible.

Absorbed serum.

These tests were carried out on the sera taken 31.1.72 and 4.2.72. The samples were pooled as only a small volume of blood was taken at each date. The plants used to give absorbing stigmas were 268, 158 and 203. All were brussels sprouts of S-genotypes S_5S_5, $S_{23}S_{15}$ and S_2S_2 respectively. Agar gels and well patterns 1 and 2 were used to test the serum against the following genotypes:

Well 1. Antiserum 8 absorbed.

2. $S_{45}S_{45}$, b.s. mixed (as used to inject rabbit).

3. 268, 158 and 203 b.s. (as used for absorption).

4. 173, $S_{45}S_{45}$, b.s.

5. As well 2.

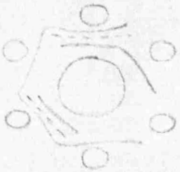
6. As well 3.

These plates were set up early in February 1972, when material was scarce. The plates were not duplicated and well 6 was loaded with stigma extract in the concentration used for absorption as opposed to the normal concentration in other wells.

The results can be seen from photograph 66. A faint but distinct band could be seen against the S_{45}-containing genotypes. The band was continuous between wells 4 and 5. A faint band was visible against well 3 very close to the antigen well and this band was also present against well 6 but was more distinct due to the stronger concentration of this extract. Well pattern 2 gave similar results to 66, but the bands were fainter. The band against wells 2, 4 and 5 appeared to be S_{45}-specific.

The serum was tested again against a wider range of S-genotypes. The serum was absorbed as described previously. Well pattern 1 was used to compare the serum against the same genotypes as previously with the addition of 271, S_2S_2 and 270, S_5S_5 brussels sprouts. The results can be seen from the diagram:

Diagram 5.



Absorption was not complete, but a single band specific to the S_{45}-genotypes was present.

The serum taken 10 days after the second booster injection was absorbed and tested as for the 31.1.72 and 4.2.72 serum. The S_{45}-specific band was again visible.

The serum taken 15 days after the second booster injection was absorbed as described for the other sera and was tested against the same genotypes with the addition of another S_{45}-containing brussels sprout line. This had just started to flower and had been found by pollination and uv microscopy to be homozygous for the S_{45}-allele. It shared the S_{45}-specific band with the other S_{45}-containing genotypes.

The serum samples taken 18 and 21 days after the second booster injection were absorbed with a different mixture of stigma extract. The lines used were the brussels sprouts 7872, 7501 and 7344 which contained S_2S_2, S_5S_5 and $S_{23}S_{15}$ respectively. The absorbed sera were tested against a wider range of genotypes than previously. The S_{45}-specific band was visible in one of the plates set up with serum collected 18 days after the injection and in both plates set up with serum taken 21 days after the injection. The reason for the lack of band in one plate could not be explained as the same batches of absorbed serum and stigma extract had been used in both plates.

In all these plates the S_{45}-specific band was rather faint and close to the serum well. This indicated a lower concentration of antibody in the serum than antigen in the stigma extract.

Conclusions.

This serum contained an S-allele-specific antibody. The antibody was not detected before the first booster injection was given, although limited tests only were carried out at the end of the year when the plant material was not at its best. The S_{45}-specific antibody was detected after the first booster injection when the only new band was that designated t_4. This band corresponded in position with the specific band.

Comparison of the response of this rabbit to booster injections with the response of rabbit 6 shows a number of differences. Firstly, the rise in titre shown by antiserum 8 occurred between 4 and 15 days after the booster injection. The rise in antiserum 6 took place sooner, between 2 and 10 days after the injection. Secondly, only one previously-undetected band was found in antiserum 8 after the first booster, and no new bands appeared after the second. In antiserum 6, three previously-undetected bands appeared after the first booster, and two after the second. Thirdly, antiserum 8 maintained its antibody titres for longer than antiserum 6. Titres had begun to fall by 17 days after the first booster given to rabbit 6, and by 14 days after the second. After the first booster given to rabbit 8, titre had not started to fall when the second booster was given seven weeks after the first. The titre of all antibodies was then maintained for at least a further three weeks after which no further bleeds were taken.

These differences could be attributed to the concentration of the stigma extracts used for the boosters. All boosters given to rabbit 6 contained 300 stigmas in 0.6 mls of saline. The first booster injection given to rabbit 8 contained 300 stigmas in 0.4 mls of saline and so was more concentrated than the booster given to rabbit 6, although in the latter case two injections were given. The second booster given to rabbit 8 contained only 200 stigmas in 0.6 mls saline and so was less concentrated than the second booster given to rabbit 6. It is more likely that the differences lie in the response of the rabbits to the stigma extracts. However, it is interesting to note that the serum raised at S.H.R.I. by Dr. Nasrallah to $S_{45}S_{45}$ brussels sprout stigma extract also showed the S-protein-specific antibody.

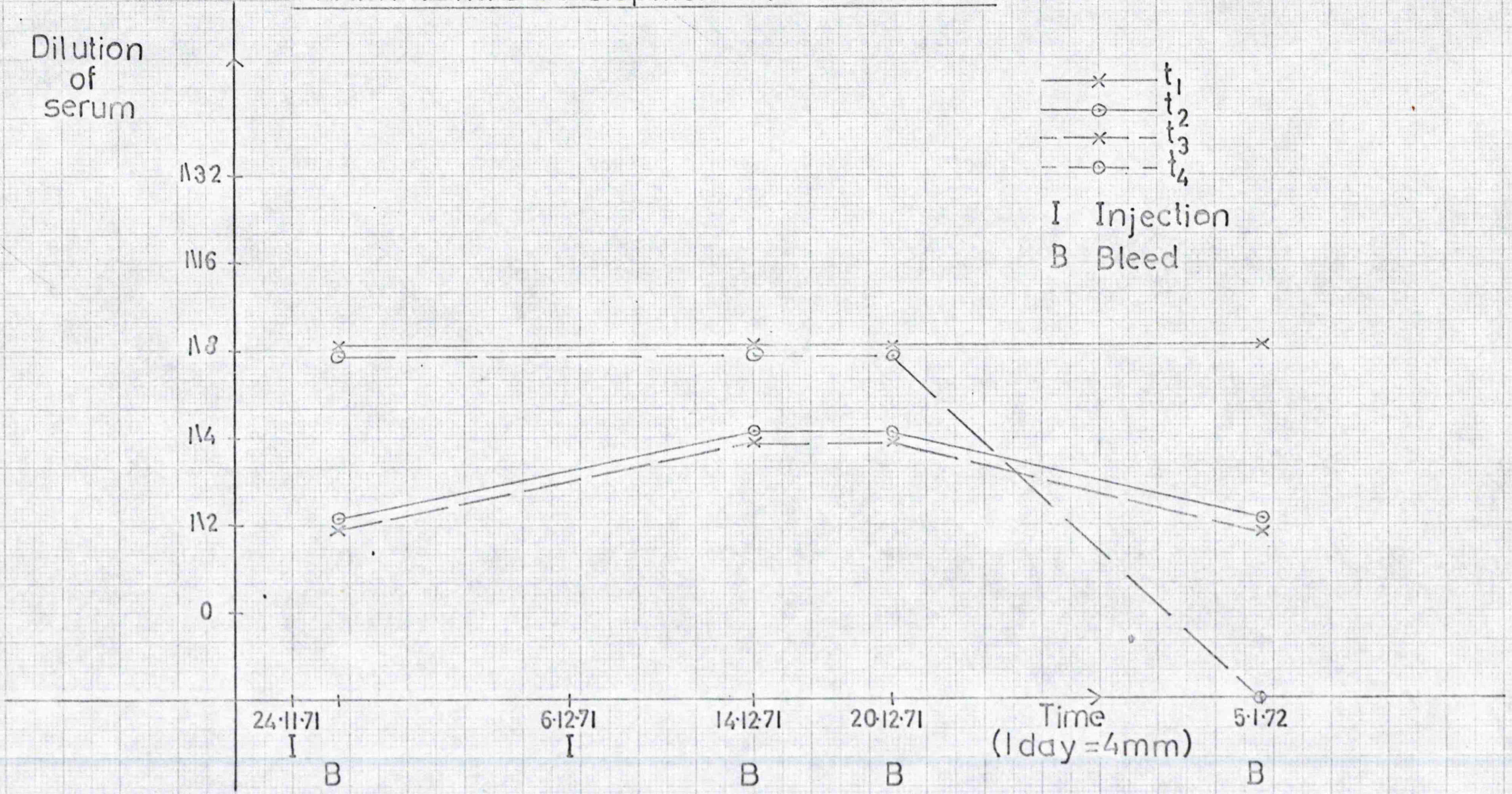
ANTISERUM 9: Formalin-treated S23, $S_{23}S_{23}$, high dominance, kale stigma extract.

Hollings and Stone, 1962, had shown that by treating Celery Yellow Vein virus with formalin to give a 0.2% concentration before injection into rabbits, they could produce an antiserum to the virus, where previous attempts with untreated virus had failed. Work with some animal viruses had previously shown that treatment with formalin destroyed infectivity without apparently affecting their antigenicity. The formalin seemed to stabilise the Celery Yellow Vein virus which previously must have been rapidly metabolised by the rabbit. This seemed to be the situation with the stigma extracts. Tests on antiserum 6 had shown that the titre reached was never high, a maximum of 1/32, and that reaction to injections was immediate and short-lived. This indicated that, despite the Freund's adjuvant, the stigma proteins did not remain in the rabbit's body to enable further antibody production, but were rapidly broken down and removed from the body.

An equal volume of 0.4% formalin solution was added to each stigma extract to give a final concentration of 0.2%. The formalin-treated extract was then left at 0°C for an hour and dialysed at 0°C for 24 hours against buffer to remove the formalin. The extract was then injected into the rabbit exactly as for other stigma extracts.

The rabbit was bled before receiving any injections. It was then given two i/v injections on consecutive days each containing 450 stigmas. The rabbit was bled 9 days

GRAPH 9. Titre development of Antiserum 9.



later and a bleed was attempted 12 days later. This rabbit was a very small animal with small ears and very narrow ear veins. Although it was healthy, great difficulty was encountered in bleeding the animal. The volume of serum obtained was never more than 2 mls, and, in this case, no serum at all was obtained. A course of i/m anf s/c injections was commenced on the same day. Ten injections were given over a period of 6 weeks and the rabbit received a total of 3,300 stigmas, by these injections. The rabbit was bled once 10 days before the end of the injection schedule and was then bled 8, 14 and 30 days after the last injection.

Dilution Tests.

No reaction was obtained with the pre-injection serum or the serum taken after the i/v injections. From graph 9 it can be seen that only four bands were present, and no antibody had a titre higher than 1/8. Some increase in titre occurred after the injection given 6.12.71, but the titre of three of the four antibodies had started to fall by 5.1.72, a month after the last injection.

Genotype Comparison Tests.

Unabsorbed serum.

A plate was set up with the serum taken 10 days before the last injection. The plate was set up in December, so material was scarce. Well pattern 3 and agar gels were used and the wells loaded as follows:

Well 1. Antiserum 9, 26.11.71.

2. Mixed kale stigma extract, $S_{27}S_{27}$, $S_{16}S_{16}$ and S_5S_5.

3. S_{23}, $S_{23}S_{23}$, k.

4. 8344/10, $S_{45}S_{2}$ and 7872, $S_{2}S_{2}$, b.s.

The results can be seen from photograph 67. Three bands were visible against the S23 stigma extract which were not present against the brussels sprout extract, and two which were not present against the mixed kale extract. This was a similar reaction to that obtained in tests carried out concurrently with antiserum 6. Tests carried out in 1972 with this serum did not show any specific genotype differences as can be seen from photograph 68. It was decided that this band was not associated with the S_{23}-protein, for the reasons outlined under antiserum 6.

Absorbed serum.

This serum was not absorbed in 1971, but the 14.12.71 serum was absorbed and tested in 1972 with the plants grown from seed provided by Dr. Thompson along with antisera 5 and 6. The serum was absorbed and tested as described for antiserum 6. Absorption was almost complete and no S_{23}-specific band was visible.

Conclusions.

Treatment of the antigen with formalin did not improve the antigenicity of the extract, rather it impaired the response. Only rabbit 1 gave a poorer response and rabbit 5 gave a comparable result. Both these had been injected with extract which had been frozen, thawed and centrifuged. No S_{23}-specific antibody was raised and the treatment was not repeated.

CONCLUSIONS FROM RABBITS 1-9

Table 2 shows a comparison of the first courses

TABLE 2

Table of comparison of the first course of injections and bleeds given to Californian rabbits 1-9 in 1971. None of these rabbits produced an S-protein-specific antibody in response to these courses.

Antiserum No.		1	2	3	5	6	8	9	7 (Pollen)
S-allele		S2	S2	S15	S23	S23	S45	S23	S23
No. of inj.		8	6	8	7	5	12	12	7
Type of inj.		4 i/v 4 i/m	6 i/m & s/c	3 i/v 5 i/m & s/c	7 i/m & s/c	5 i/m & s/c	2 i/v 10 i/m & s/c	2 i/v 10 i/m & s/c	7 i/m & s/c
Period of inj. (days)		42	17	39	28	16	43	69	29
State of inj. extract		Frozen	Fresh	Frozen	Frozen	Fresh	Fresh	Formalin treated	Fresh
Average concn. of inj. (stigs/ml)		600	1000	1000	550	550	700	650	Pollen from 1000 fls/ml
Total no. of ab. detected		2	6	5	4	3	5	4	6
Highest titre reached x days after the last inj.	x=10	1/8		1/16	1/8		1/32	1/8	
	x=20	1/8	1/32	1/32	1/8		1/32	1/8	1/8
	x=30		1/32	1/16	1/8		1/16	1/8	1/16
	x=40		1/32		1/8	1/32			1/16
	x=50		1/32			1/32			
Highest overall titre reached		1/8	1/32	1/32	1/8	1/32	1/32	1/8	1/16

of injections and bleeds given to rabbits 1-9 and of their responses. A comparison of the booster injections is given in table 3.

The first conclusion to be drawn from the work carried out in 1971 and early 1972 was that S-protein is not very antigenic in rabbits. None of the first courses of injections stimulated the production of an S-protein-specific antibody. As a result of this, the titre buildup to the booster injections given to rabbits 6 and 8 was followed more closely by regular bleeds. It was found that booster injections stimulated more response in a sensitised rabbit than that produced by the first course of injections, including in two cases the production of an S-protein-specific antibody. The booster injections also showed that the injection extract does not necessarily need to be very concentrated, as lower concentrations were used than for most of the initial injection extracts. This is also shown by a comparison of the treatments given to rabbits 2 and 6. Rabbit 2 received injections which were almost twice as concentrated as those given to rabbit 6. The period and number of injections was almost the same in the two cases, yet both produced the same overall titre. However, rabbit 6 produced fewer detectable antibodies, but was less healthy and was not bled so soon after the cessation of injections as rabbit 2. Some antibodies may, therefore, have been missed.

The variation in the response of different rabbits to the injections of stigma extract is also evident from tables 2 and 3. The booster injections given to rabbits 6 and 8 show this particularly. Rabbit 8 showed a slower titre buildup than rabbit 6, but maintained its titre for longer. Also, fewer previously-undetected antibodies appeared in rabbit 8 serum after the boosters than in rabbit 6 serum. It is well known that rabbits vary greatly in their response to immunisation (Boyd 1966), and this is particularly noticeable in this case where the protein in question is not very antigenic.

From an examination of table 2, it can be seen that stigma extract which has been frozen, thawed and centrifuged or treated with formalin produces a poorer antiserum response than freshly-prepared stigma extract. In all cases except antiserum 3, lower titres were stimulated by such treatments. Also, both rabbits which produced S-protein-specific antibodies were injected with fresh extract.

The length of the injection schedule did not appear to affect the response of the rabbits. Rabbit 2 received a short injection schedule of 17 days, but produced more detectable antibodies and as high an overall titre as rabbit 8 which was injected over a period of 43 days. The inclusion of i/v injections in the schedules did not appear to affect the overall response either, although no reaction was detected in any batch of serum taken after a course of i/v injections. Tests on antiserum 7 showed that pollen proteins were more antigenic than stigma proteins in that they gave much clearer and well-defined antigen-antibody reactions.

Since the stigma proteins were evidently so weakly antigenic it was decided that frequent injections such as twice- or thrice-weekly may be more effective in maintaining titre. Frequent bleeds would enable the development of titre to be followed more closely so that the best injection procedure could be determined.

CHAPTER 9.

RABBITS INJECTED IN 1972.

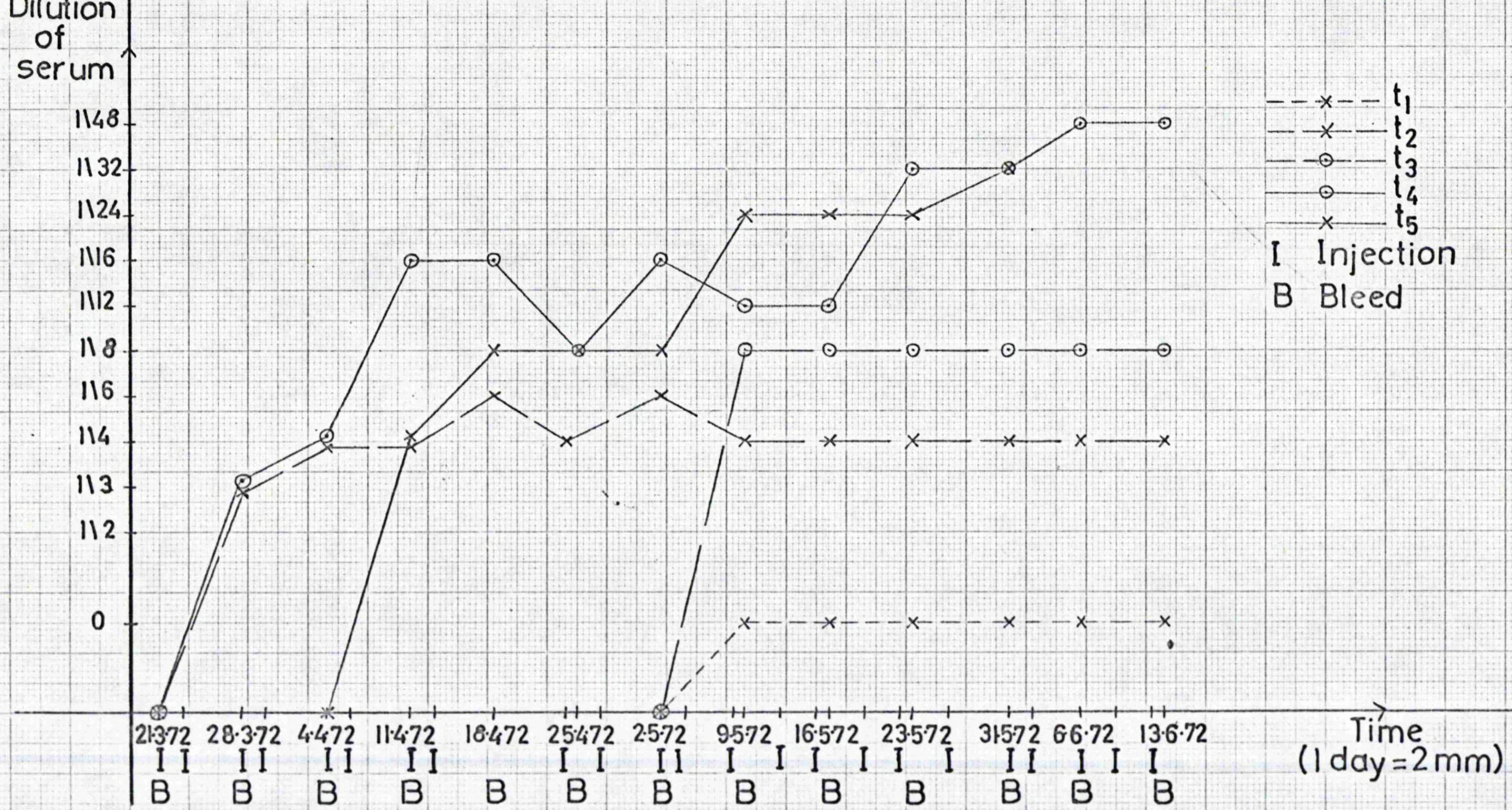
In March 1972, four rabbits were injected in the hope that one would prove as satisfactory as rabbits 6 and 8 for antibody production to *Brassica oleracea* S-protein, but would give a higher titre. The rabbits were injected concurrently so that all received stigma extracts from flowers which were from the first period or flush of flowering of 1972. This was because the plants are most vigorous at this time of year, and there is no possibility of complications such as end-of-season compatibility. Another reason was that all the plants used had been tested for their S-allele constitution by pollination and uv microscopy just prior to the start of injections. Also, most flowers are produced during the first flush, so it was certain that sufficient suitable flowers would be available for preparation of stigma extract. The flowers chosen were, as far as possible, those which had opened on the day of collection and so were unlikely to have been contaminated with foreign pollen.

In accordance with the conclusions drawn from the work done in 1971, injections were to be given twice-weekly or thrice-weekly and bleeds were to be taken at weekly intervals. Each twice-weekly injection was to contain, as far as was possible, 200 stigmas extracted in 0.8 mls of saline, and each thrice-weekly, 100 stigmas in 0.4 mls saline. Extracts were to be introduced by i/m injection, s/c injection or a combination of the two. i/v injections were to be given to one rabbit only.

Preliminary tests on the serum were by genotype comparisons on the absorbed serum. If any rabbit was found to have raised a serum containing an S-protein-specific antibody, then this rabbit was to receive further injections and bleeds. This was in the hope that further regular injections would increase the titre of the S-antibody. The S_{45}-antibody raised in rabbit 8 had a low titre and results were not consistent. This method of increasing the titre was planned as a result of the work by Fulton (1967 a & b, 1968) on antiserum production to labile viruses. He found that an antiserum could be raised to Rose Mosaic virus only by giving i/m injections every three days. As injections were continued on this regular basis. the titre continued to rise. Fulton then went on to raise antisera to other labile viruses including Cherry Necrotic Ringspot, Plum Line Pattern and Apple Mosaic viruses, by the same method. He found that suitable titres of 1/640 were obtained after as few as seven injections but that titres as high as 1/2560 were obtained after additional injections. Fulton also raised an antiserum to healthy tobacco leaf protein with a titre of 1/1280. This was also raised by frequent injections, and bleeds were taken while injections were still being given.

Of the four rabbits injected, two were of the New Zealand White (N.Z.W.) breed and the other two were Californians. The two breeds were to be compared, as far as possible with the limited numbers used, for their capacity to raise an S-protein-specific antibody. The overall titre response of the two breeds was also to be compared. The N.Z.W. rabbits were injected with

GRAPH 10. Titre development of Antiserum 10.



7872 and 7501, the Californians with 224 and S14.

Genotype-comparison tests were not carried out on the unabsorbed sera as such tests had not revealed the S_{45}-specific band in antiserum 8. Also, the number of tests which could be carried out was limited by time and availability of flowering material. Agarose gels and well pattern 1 were used for all genotype comparison tests.

ANTISERUM 10: 224, $S_{16}S_{16}$, high dominance, kale stigma extract.

After the pre-injection bleed, this Californian rabbit was given eight injections, some i/m, some s/c and some a combination of the two. It received 1,600 stigmas, as each injection contained 200 stigmas. The injections were given twice-weekly. Bleeds were taken at weekly intervals, and a test on the serum taken 21 days after the first injection gave promising results. The injections were continued as a result of this and the rabbit received twice-weekly injections as already described for a further seven weeks after which the rabbit died of anaphylactic shock. There was a gap of 11 days between the last of the first course of injections and the first of the continuation injections.

Dilution Tests.

As can be seen from graph 10, the serum taken before any injections were given showed no antibodies to the stigma extract. Intermediate dilutions were made of each serum as well as the usual dilutions so as to follow the titre development in more detail. As injections were continued, titre of all antibodies increased until the gap in the injections between 13.4.72 and 24.4.72. In the serum taken 25.4.72, two of the three antibodies had fallen in titre, although the rabbit had been re-injected on 24.4.72. This shows that, in the early stages of injection at least, titre very soon falls if regular injections are not maintained. Also, there is a lag period between the cessation of injections and the fall in titre of 10 days, and between the recommencement of injections and the increase in titre of 8 days. After the recommencement of injections, the serum also became more complex, five bands were visible where only three were visible before. All five bands remained until the death of the rabbit and the titre of two continued to rise with further injections until it reached 1/48. This was the highest titre recorded from any rabbit injected with stigma extract.

Although three bands were consistently visible on dilution plates between 11.4.72 and 25.4.72, and five between 2.5.72 and 13.6.72, it was suspected that some of these latter bands were made up of more than one component. The banding pattern from no two bleeds was identical, different bands were prominent in different plates and sometimes bands appeared to split at some dilutions. These effects were not sufficiently consistent to be recorded.

Genotype Comparison Tests.

Absorbed sera.

The first serum to be tested was that taken

11.4.72, 21 days after the first injection. The serum was absorbed with an extract containing the following kale stigmas: 221, S_2S_2; 222, S_5S_5 and S21, $S_{21}S_{21}$. The following plate was set up with agarose gels and well pattern 1:

Well 1. Antiserum 10, 11.4.72, absorbed.
2. 224, $S_{16}S_{16}$, k.
3. S14, $S_{14}S_{14}$, "
4. Absorbing kales.
5. 224, $S_{16}S_{16}$, k.
6. S23, $S_{23}S_{23}$, "
7. 224, $S_{16}S_{16}$, "

The results can be seen from photograph 69, which in fact represents the 16.5.72 serum, but the result was the same. All antibodies had been absorbed except one, and this produced a band against wells 2, 5 and 7, which were those containing 224 stigma extract.

The above procedure was repeated, and the same result obtained. Plates were also set up to measure the titre of the S_{16}-band. Well pattern 6 was used. This was a reversal of well pattern 1, so that the relative concentrations of antiserum and stigma extract were the same as in the genotype comparison tests. The absorbed serum was diluted by twofold steps with saline and the following plate set up:

Well 1. 224, $S_{16}S_{16}$, k.
2. Antiserum 10, 11.4.72 absorbed.
3. " " 1/2
4. " " 1/4
5. " " 1/8

From photograph 70, it can be seen that the S_{16}-band was visible to a dilution of 1/4, but that absorption was not complete. Two other faint bands were also visible. These were also visible in one of the genotype comparison plates, against the other genotypes as well as 224, but the S_{16}-specific band was distinct from these.

It appeared from these preliminary tests that this rabbit may have produced an S_{16}-specific antibody. All other serum samples taken from the rabbit were tested by absorption and genotype comparison tests. The method was essentially as described except that the genotypes in the mixture of absorbing stigmas were varied, as were the genotypes in wells 2-7. This was to check that the same result was obtained whichever combination of kale stigmas available was used. Unfortunately, no other source of the S_{16} allele was available and none has been found since, so the 224 kale could never be compared with other S_{16}-containing stigma extracts except its own progeny. At this stage, therefore, the possibility that the band seen was produced by a protein, totally unrelated to the incompatibility mechanism, present in the 224 kale, but in no other kale flowering that season, could not be dismissed.

The earliest serum sample in which the band was detected was that taken 4.4.72, 14 days after the first injection. The band present against the 224-containing wells was continuous between them but was very close to the antiserum well, and rather faint. This indicates a lower titre of the S_{16}-antibody in this serum than in that taken a week later on 11.4.72.

The S_{16}-antibody was also present in the sera taken 18.4.72 and 25.4.72, but the reaction obtained with the 25.4.72 serum was similar to that with the 4.4.72 serum, indicating that the titre of the S_{16}-antibody had fallen.

In none of the tests described was absorption complete. Other faint bands were visible against most genotypes but the S_{16}-band was always quite distinct from these. An example of this is seen in photograph 71, where the 9.5.72 serum was absorbed 3:1.5 with an absorbing mixture containing 36 stigmas in 0.1 mls saline. This gave more absorption than that used in the original test on the 11.4.72 serum and other non-specific bands were still visible. This was because the titres of the other non-specific antibodies were rising as can be seen from the graph. One of these non-specific bands, against well 3 containing 222, S_5S_5, was particularly distinct and showed identity with a band present against the 224-containing well. The S_{16}-band, however, formed an extension beyond the 222 band, showing that the S_{16}-specific antibody was present in the serum. What appeared to be a single band against the 224 well was, in fact, two, one of which was shared with the 222 extract and possibly with the absorbing stigma extract in well 4.

With later bleeds, absorption was increased further, the 16.5.72 serum was absorbed 3:2.5 and the 23.5.72 serum 1:1. It must be remembered, that as well as increasing the absorption of non-specific proteins, the serum was also being diluted further by increased absorption volumes.

A change occurred in the serum between 16.5.72 and 23.5.72. This can be seen from photographs 69 and 72. 69 shows the 16.5.72 serum absorbed 3:2.5 and 72 shows the 23.5.72 serum absorbed 1:1 with the same mixture of absorbing stigmas. Wells 2-7 were identical also. Firstly, despite the larger absorption volume used for the 23.5.72 serum, the band against the 224-wells was far more distinct than with the 16.5.72 serum. Also, there was a band visible against the S14 in well 3 in 72, which was not visible in 69. This band was diffuse and not at all clear, but it showed definite identity with part of the band against the 224 extract. However, the S_{16}-band formed a faint but definite extension beyond the band against the S14 extract. It appeared that titre may have been built up to detectable levels of an antibody to a protein shared by 224 and S14 kales, although this was not apparent from the dilution plates on the unabsorbed sera.

An identical reaction was produced under the same conditions by the 31.5.72 and 6.6.72 sera as seen in photographs 73 and 74 respectively. The serum taken 13.6.72, which was the last bleed, was absorbed 3:1 with the same absorbing mixture to compare the results with the almost total absorption produced with earlier bleeds such as 11.4.72 absorbed 3:1. The result can be seen in photograph 75, where a band was present against each genotype and showed identity with part of the S_{16}-band. The latter, however, produced extensions beyond the other band. It was evident that what appeared to be one band against the 224 well was, in fact, more than one band. In none of the later sera was absorption complete.

As a result of the above findings, three batches of the serum taken 31.5.72 were absorbed. One was absorbed with a mixture containing 7872, 221, S23 and 222 stigmas, one with a mixture containing all these genotypes plus S14, and the other with the same mixturw including 224 rather than S14. The total number of stigmas was always the same and serum was absorbed 1:1. Well pattern 6 was used, with 224 stigma extract in the centre well and dilutions of the absorbed serum to 1/8 in the surrounding wells. The plates set up with 224 in the absorbing mixture showed no bands. The results of the other two absorption mixtures can be seen from photographs 76 and 77. The serum absorbed normally gave one bright band visible to a dilution of 1/4. The serum absorbed with mixture containing S14 gave two faint bands, both of which were visible to a dilution of 1/2. Absorption with the mixture containing S14 stigmas removed considerably more than the normal absorption mixture. This was in agreement with the previous results that the apparently single bright band left after absorption with a mixture not including S14, may in fact, consist of more than one component.

Diffusion of proteins from intact stigma and pollen tissue.

The method for extraction of pollen proteins in Coca's fluid has been described under antiserum 7. At the sage time as those tests were done, similar plates were set up with antiserum 10 to test whether either of the faint bands produced against the pollen antiserum was continuous with the specific band produced after absorption of the antiserum 10 taken 31.5.72. The following plate was set up using agar gels and well pattern 1.

Well 1. Antiserum 10, 31.5.72, absorbed.
2. 224 stigma macerate.
3. 224 pollen extract 100,000 Noon units /ml.
4. " " " 1/2 50,000 Noon units/ml.
5. " " " 1/4 25,000 " "
6. " stigma macerate.
7. " pollen " 200,000 " "

The results can be seen from photograph 78, the S_{16}-specific band was visible against the 224 stigma macerate only. It was not present against either the 224 pollen extract or macerate. This shows that the S_{16}-protein was not present in either pollen preparation.

Coca's fluid was also used to extract the proteins from intact stigmas. It should be pointed out that the stigmas had one cut surface where they had been attached to the style. Since the Coca's fluid produced such poor results with the pollen, another batch of stigmas was extracted in saline of pH 6.5. The stigmas were extracted for 48 hours at 21°C and 24 hours at 0°C. The following plate was set up using agar gels and well pattern 1:

Well 1. Antiserum 10, 31.5.72, absorbed.
2. 224 macerate.
3. " extract in saline.
4. " " " Coca's fluid.
5. " macerate.

From photograph 79, it can be seen that the S16-protein was present in both macerate and extract in saline, but not in the extract in Gown's fluid. There were three differences between the two media, the pH, the sodium bicarbonate and the phenol. The most likely factor affecting protein diffusion is the pH, as shown by Stanley (1971) with pollen proteins.

From this it would appear that the S-protein diffused freely from the intact stigmas. This was also shown in an experiment set up in conjunction with T.Hodgkin. He found that a cap of gelatin placed on the stigma before pollination resulted in germination and tube growth irrespective of the genotype of pollen and stigma. An experiment was set up to test whether or not the S-protein diffused into the gelatin. This tested the hypothesis that the S-protein either does not diffuse into the gelatin, or is so diluted after the diffusion that it cannot exert the inhibitary action on the pollen which is associated with its presence. A small cap of gelatin completely covering the surface of the stigma was applied to 30 224 kale flowers. As a control, gelatin caps were also applied to the ends of 10 matchstalks. After 24 hours the gelatin caps were removed with care so that no stigma tissue adhered to the gelatin. A gel was set up using well pattern 1 to compare the S-protein content of 10 gelatin caps with the 224 stigma macerate against absorbed antiserum 10 taken 31.5.72. The 10 caps were placed in a well and the well topped up with saline to give a suitable diffusion medium between the gelatin and the agar. The same was done with the 10 caps from the matchstalks.

Dilutions of the stigma macerate up to 1/4 were prepared and the following plate set up:

Well 1. Antiserum 10, 31.5.72, absorbed.

2. Gelatin caps from 10 224 stigmas.

3. 224 macerate 1

4. " " 1/2

5. " " 1/4

6. Gelatin caps from 10 224 stigmas.

7. " " " " matchstalks.

The results can be seen from photograph 80. A faint band was present against both wells containing gelatin caps from the 224 stigmas. This band was continuous with the S_{16}-band against the 224 macerate and its dilutions. No band was visible against the well containing caps from the matchstalks. This band was about the concentration of the 1/4 dilution of the stigma macerate which is approximately as expected. If the gelatin cap is assumed to be of roughly the same volume as the stigma, then assuming free diffusion, the stigma and gelatin cap should both end up with half the normal stigma concentration of S-protein. As 10 caps were placed in one well, this is equivalent to the S-protein content of 5 stigmas. One well contains 0.05 mls of extract. Stigmas were extracted in the proportion of 25 stigmas in 0.1 mls of saline, so a 1/4-strength dilution contains the equivalent of the S-protein content of 3.175 stigmas. Thus, the concentration of the band produced from the gelatin caps would be expected to be between 1/2 and 1/4-strength dilution. This is assuming no further production of S-protein by the stigma after the concentration had been reduced by diffusion into the gelatin cap.

Conclusions.

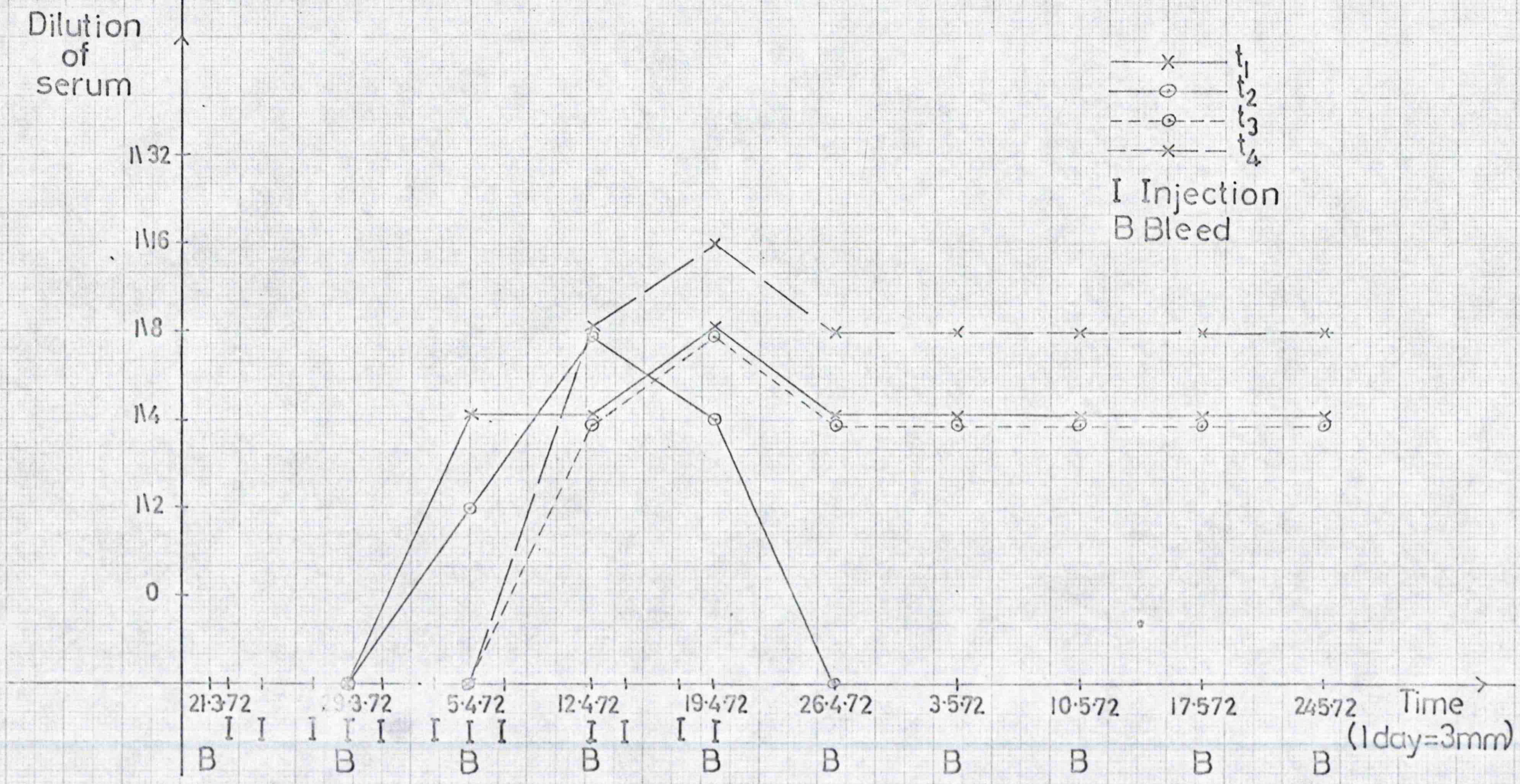
It appeared that as injections were continued, a specificity was built up which was shared by S16 and S14 stigmas and which precipitated in the same area of gel as the S_{16}-protein-antibody complex. However, Landsteiner 1936 stated that as immunisation is continued, the specificity of an antibody often apparently becomes less and an increase in the strength and extent of cross-reactions occurs. Also, Hooker and Boyd 1941 found that qualitative changes in the combining power of an antibody can result from increased immunisation. They showed that an antibody produced during an extended injection period could combine with a related antigenic determinant with which the antibody at first showed no reaction. This may have been the situation with the S_{16}-protein-antibody. A cross-reaction may have been produced with a protein in the S14 stigmas which had previously been unable to react with the S-protein-antibody. As immunisation was continued, and specificity lost the S_{16}-protein stimulated new specificities with which the protein in the S14 could combine. The nature of the protein in the S14 stigmas was unknown, and no other S_{14}-containing plants were available at S.H.R.I., so the possibility of a cross-reaction with the S_{14}-protein could not be tested. This would also explain why no new specificity was detected in the dilution plates.

The S_{16}-band obtained in genotype-comparison tests corresponded to band t_4 of the dilution plates (see graph 10). The evidence for this is firstly that absorption of 4.4.72. serum had revealed a single S_{16}-specific band. This must have been one of the two bands visible in the dilution plates, assuming that both consisted of one component only. Secondly, the position of band t_4 in well pattern 2 corresponded with the position of the S_{16}-protein band in well pattern 1.

The third piece of evidence is derived from observations on the progress of the band in later bleeds compared with the genotype comparison plates. The antibody undoubtedly increased in titre, this can be seen from the dilution plates set up to measure the titre of the S-protein-antibody. For example, photograph 70 shows the 11.4.72 serum absorbed 3:1. Photograph 76 shows the 31.5.72 serum absorbed 1:1. Both sera had a titre of 1/4, but the 31.5.72 serum sample was absorbed with a larger volume of extract and so the serum was diluted to half the strength of the 11.4.72 serum. The S-16 antibody in the 31.5.72 serum, therefore, was at one twofold dilution higher than that in the 11.4.72 serum. This agrees with the observations on the dilution plates where band t_4 had a titre of 1/16 on 11.4.72 and 1/32 on 31.5.72. The titre chart of band t_2, the only other band visible in the 4.4.72 serum does not follow this pattern.

Other changes in the titre of band t_4 are reflected in the genotype comparison plates. For example, the specific band in the 25.4.72 serum was faint and close to the antiserum well indicating the fall in titre exhibited by band t_4. Also, the rise in titre of the

GRAPH 11. Titre development of Antiserum 11.



S-protein between 16.5.72 and 23.5.72 can be seen in band t_4 between these dates.

The diffusion of the S_{16}-protein into the gelatin caps on the stigmas did not prevent the germination of the plant's own pollen. This indicates one of three possibilities. Firstly, the S-protein as detected by these methods is not the factor directly involved in inhibition of pollen germination. Secondly, it may be involved in inhibition, but cannot function without some factor in the papillae cells which either does not diffuse into the gelatin or does not function outside the cell. Thirdly, the S-protein cannot exert its effect when diluted, but must be present at some threshold level as suggested by Sampson (1960). What is evident, however, is that the S-protein as detected by these means diffuses freely into a suitable medium placed over the stigma.

ANTISERUM 11: 7872, S_2S_2, intermediate dominance, brussels sprout stigma extract.

The rabbit was a New Zealand White and was bled before receiving any injections. It then received a total of 1200 stigmas by thrice-weekly i/m or s/c injections over 4 weeks. The rabbit was bled at weekly intervals during the period of injections and for 5 weeks after the injections had ceased.

Dilution Tests.

As can be seen from graph 11, no bands were detected in the pre-injection serum or in the serum taken one week after the first injection. From week two, titre rose up to a maximum of 1/16 on the day of the last injection. After injections were ceased, titre of all antibodies fell, but stabilised for the rest of the 5 week period during which bleeds were taken.

Genotype Comparison Tests.

Absorbed sera.

The sera taken 12.4.72, 19.4.72, 26.4.72, 3.5.72, 10.5.72 and 17.5.72 were absorbed with absorbing extracts made up of a wide range of genotypes. All absorbed sera were tested against stigma extract of 7872 and 221, which is an S_2S_2 kale. A brussels sprout heterozygous for S_2, called 143 was also included where possible, as well as a range of non-S_2-containing genotypes.

The 12.4.72 serum was absorbed and tested as described above and the results can be seen from photograph 81. Absorption was not complete and no specific band was visible against the S_2-containing genotypes in wells 2, 3 and 6. As a result of this the serum was absorbed again using a more concentrated extract of 35 stigmas in 0.1 mls of saline in the proportion of 3:2. A single faint band was visible against all wells, so it was not S_2-specific.

Photograph 82 shows the results of absorption of the 19.4.72 serum. A continuous band was visible against wells 2, 3 and 4. Wells 2 and 3 contained S_2-genotypes, well 4 did not. The band was also visible against the 7872 in well 6. Again it would appear that this band was not S_2-specific, although it could possibly have been made up of more than one component, one of which was specific.

When the serum taken 26.4.72 was tested, results were obtained as in photographs 83 and 84. In both, a single faint band was present against 7872 in wells 2 and 6, the 221 in well 3 and a very faint suspicion of a band against well 4 which contained 143, a brussels sprout of S-genotype S_2S_{45}. A fainter band against the genotype heterozygous for the S_2-allele would support the previous indication that the heterozygote has less of a particular S-protein than the homozygote. This band was specific to the S_2-containing genotypes, but was extremely faint, indicating a very low titre of the S-protein-antibody.

The 3.5.72 serum also showed a band present against the 7872 in well 2 and the 221 in well 3, but not against the 143 in well 4 (see photograph 85). However, the band appeared to curve round toward well 4. Again this could be an S_2-specific band but it was very faint.

The 10.5.72 serum also gave a banding pattern similar to that described previously except that the 7872 and 221 in wells 2, 6 and 3 were the only S_2-containing genotypes (see photograph 86). Here the band was even fainter than with the 26.4.72 and 3.5.72 sera. When this procedure was repeated, however, with a slightly different mixture of absorbing stigmas, a rather puzzling result was obtained as can be seen in photograph 87. The band against the 7872 in wells 2 and 6 was clear, but the band against the 221 in well 3 formed an extension beyond the 7872-band. There was no other S_2-containing genotype in the gel. The 7872 band did not form an extension beyond the 221 band, but the 221 band appeared to be unchanged after meeting the 7872 band, as though it were a totally different antigen-antibody system. It is possible that the different absorbing mixture had not absorbed a non-S_2-specific band which was masking the S_2-specific band.

Absorption of the 17.5.72 serum gave the same result as the 10.5.72 serum with the same absorbing extract as used to produce the 87 plate. This can be seen from photograph 88.

Conclusions.

This rabbit responded very slowly to the injections. If the response is compared with that of rabbit 10, two main differences can be seen. Firstly rabbit 11 produced no detectable response to injections until the bleed taken after two weeks of injections. Rabbit 10 showed the presence of two antibodies in its serum after only one week of injections. Secondly, the maximum titre of 1/16 reached by antiserum 11 was achieved after 4 weeks of injections. A titre of 1/16 was reached in antiserum 10 after 3 weeks of injections. Rabbit 11 appeared to react more slowly than rabbit 10 to the injections although after 5 weeks rabbit 11 had produced four antibodies to rabbit 10's three. Rabbit 11 received 300 stigmas per week via three injections, whereas rabbit 10 received 400 stigmas per week via two injections. This may be the cause of the differences.

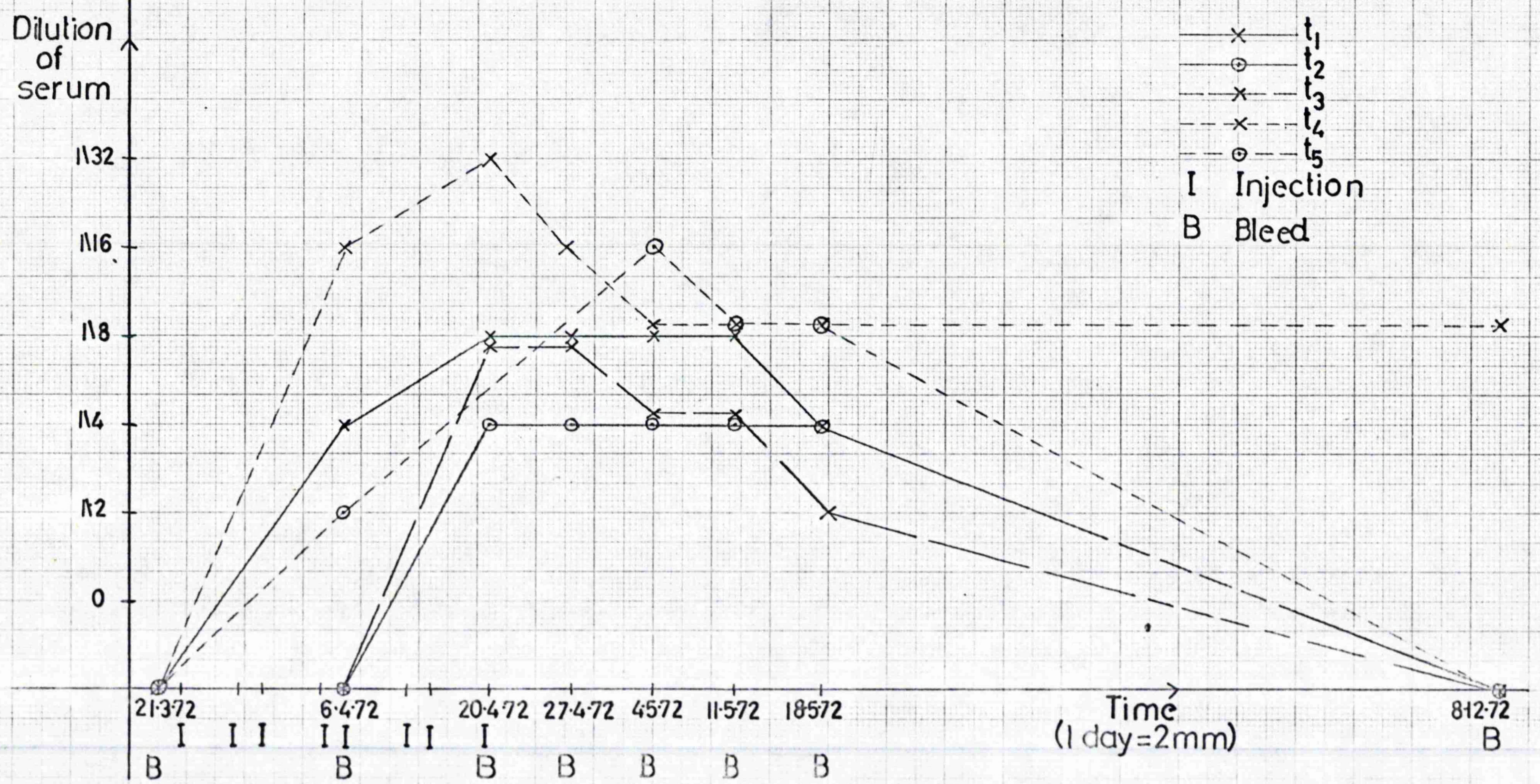
The conclusions drawn from the absorption tests are rather confused. No specific antibody was apparent in the sera taken 12.4.72 or 19.4.72, but a positive reaction was obtained with the sera taken 26.4.72, 3.5.72 and 10.5.72. Later tests, however, put doubt on the 10.5.72 serum and also the 17.5.72 serum, both of which had a 7872-specific band which did not appear to react satisfactorily with the 221. It is possible that this was due to incomplete absorption. The serum may have an S_2-specific antibody, but its titre appears to be so low as to make the results unreliable. If injections had been continued, The S-antibody may have become easier to detect. As with antiserum 10, however, absorption volumes would also have to have been increased.

From graph 15 it can be seen that the three antibodies present in the sera taken between 26.4.72 and 17.5.72 were also present in the 12.4.72 and 19.4.72 sera. There is no reason, therefore, why the possible S_2-antibody should not have been detected in these two sera. The titre of the three bands in the 12.4.72 serum was the same as in the later sera. The titre in the 19.4.72 serum was higher. The discrepancy probably lies in the protein contents of the extracts or the water content of the gels. Slight variations in these may have made the difference between precipitation or not of this antigen/antibody system.

ANTISERUM 12: 7501, S_5S_5, low dominance, brussels sprout stigma extract.

This rabbit was a New Zealand White and was given a pre-injection bleed. It then received 1600 stigmas by twice-weekly i/m and s/c injections over 4 weeks. The rabbit was bled at fortnightly intervals during the

GRAPH 12. Titre development of Antiserum 12.



injection period and at weekly intervals for 4 weeks after the injections were ceased.

Dilution Tests.

From graph 12 it can be seen that no antibodies were detected in the pre-injection serum. 14 days after the first injection a titre of 1/16 was detected which rose to 1/32 at 28 days but then started to fall when injections were ceased, as did the titre of all the other antibodies. A maximum of 5 antibodies was detected. Eight months after injections had ceased, one antibody was still detectable at a titre of 1/8.

Genotype Comparison Tests.

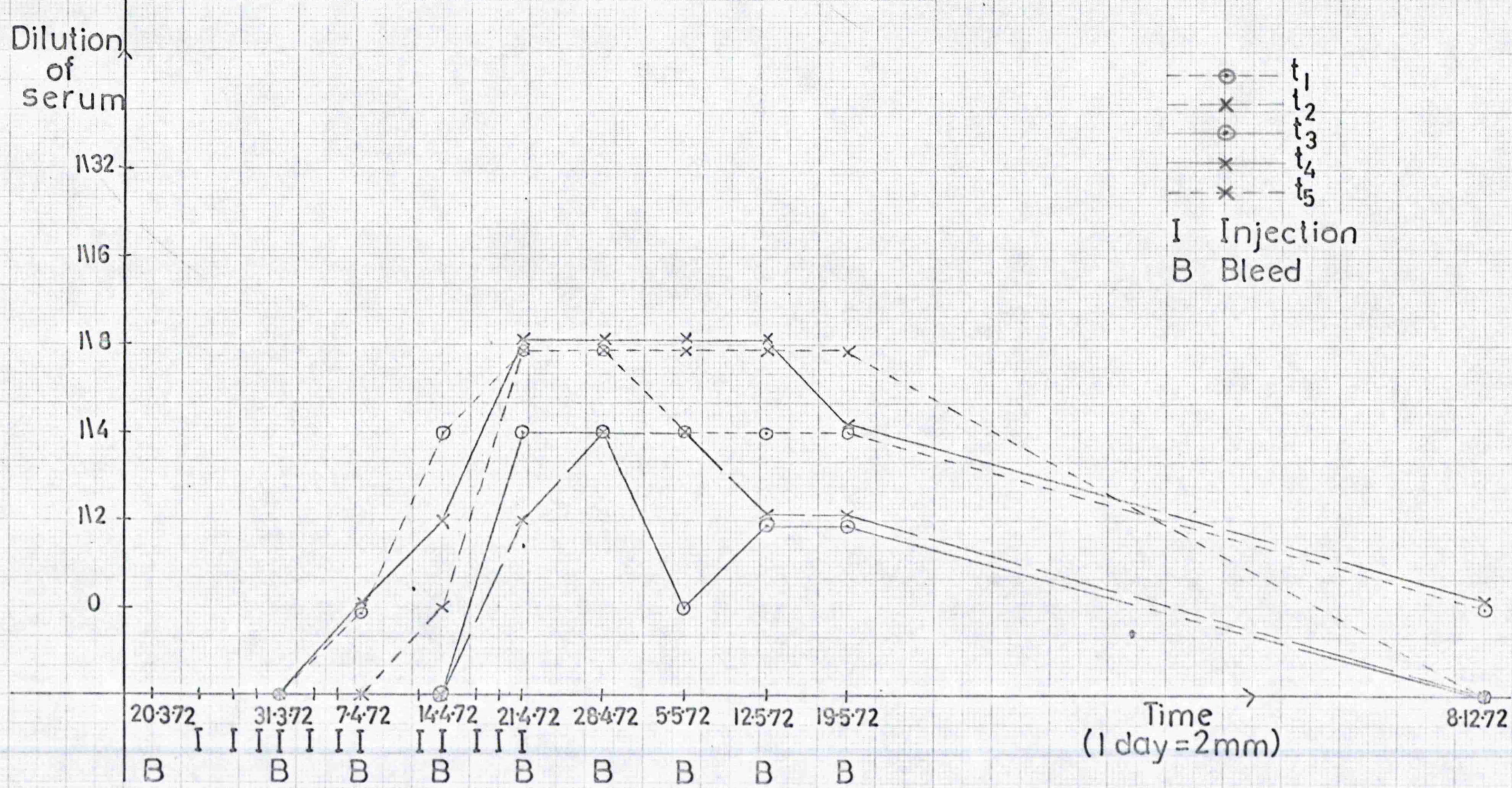
Absorbed sera.

These were carried out on the sera taken 20.4.72, 27.4.72, 4.5.72, 11.5.72 and 18.5.72. Stigmas from a wide range of genotypes were used to prepare the absorbing mixture. All absorbed sera were tested against a range of genotypes including 222, a kale homozygous for the S_5-allele.

On absorption of the sera taken 20.4.72 and 27.4.72, a pattern as seen in photograph 89 was produced. Absorption was not complete, but on further absorption of the 20.4.72 serum of 1:1 with an extract containing 35 stigmas of 0.1 mls of saline, a single faint band was visible against all genotypes.

Absorption of the 4.5.72 and 11.5.72 sera gave a pattern as seen in photograph 16. Again absorption was incomplete. The 18.5.72 serum gave a similar pattern but all bands were fainter, photograph 90.

GRAPH 13. Titre development of Antiserum 13.



Conclusions.

This rabbit showed a very rapid titre buildup, reaching 1/16 14 days after the first injection. Rabbits 10 and 11 had reached a titre of only 1/4 after 14 days. A titre of 1/32 was detected after 28 days but this was not maintained when injections were ceased. This rabbit appeared to be very sensitive to the stigma proteins and comparison with rabbit 11 showed that the New Zealand White breed of rabbit was as variable as the Californian in titre response.

No S_5-specific antibody was detected in the serum.

ANTISERUM 13; S14, $S_{14}S_{14}$, high dominance, kale stigma extract.

Rabbit 13 was a Californian and was the only rabbit injected in 1972 to receive i/v injections. After the pre-injection bleed it received three i/v injections, each containing 100 stigmas at two-day intervals. The schedule was then continued with 9 i/m and s/c injections over a period of 4 weeks. Each injection contained 100 stigmas, so the rabbit received an overall total of 1,200 stigmas. The rabbit was bled at weekly intervals during the schedule and for 4 weeks after the injections had been ceased.

Dilution Tests.

From graph 13 it can be seen that the response to injections was very poor. No antibodies were detected until 14 days after the first injection when two were present, both with a titre at no dilution. The maximum titre reached was 1/8 after 28 days when a maximum of 5 antibodies was detected. Titre of all antibodies then slowly started to fall, and 8 months after injections had ceased, two antibodies were detectable at a titre at no dilution.

Genotype Comparison Tests.

Absorbed sera.

The sera taken 14.472, 21.4.72 and 19.5.72 were absorbed with and tested against a range of kale genotypes, but there was no other S_{14}-containing genotype with which to compare the S14.

On absorption, the 14.4.72 and 19.5.72 sera gave a single faint band continuous against all genotypes tested. The 21.4.72 serum gave more faint bands, but none was specific to the S14. Further absorption of 3:2 with an extract containing 35 stigmas in 0.1 mls of saline resulted in complete absorption of all bands.

Conclusions.

Of the four rabbits receiving the first course of injections in 1972, rabbit 13 gave the poorest response. Titre buildup was slow and the highest titre reached was 1/8. This poor response was probably due to the fact that the first three injections were i/v whereas the other three rabbits received i/m injections during the first week. This is in agreement with results from other antisera where bleeds taken after courses of i/v injections have shown no detectable titre. No stimulatory effect of sensitisation by i/v injection was detected here, although the injection course may not have been sufficiently long to show this.

As a result of the absorption tests, no S_{14}-specific antibody was detected.

CONCLUSIONS FROM RABBITS 10-13.

Table 4 shows a comparison of the courses of injections and bleeds given to rabbits 10-13 in 1972 and of their responses. As already stated, the i/v injections given to rabbit 13 produced a poorer response than the courses of i/m injections given to rabbits 10,11 and 12. The inclusion of i/v injections in the course was a disadvantage in all respects. The New Zealand White breed of rabbit appeared to be as variable as the Californian in both titre response and production of S-protein-specific antibody. Rabbit 11 showed a very slow response to injections but produced an S-protein-specific antibody. Rabbit 12 gave a rapid, and relatively strong response, despite the fact that it received only 8 injections. However, it produced no S-protein-specific antibody.

Rabbit 10 received 24 injections, but the maximum titre reached was only 1/48. The extra injections had increased the overall titre but by very little compared with the effort involved in preparation and administration of the vaccine. Also, results from the later bleeds were confused by the apparent reduced specificity of the S-antibody. Rabbits 10 and 12 received twice-weekly injections of the extract of 200 stigmas. Rabbits 11 and 13 received thrice-weekly injections of the extract of 100 stigmas. The former course stimulated higher overall titres, but both courses produced an

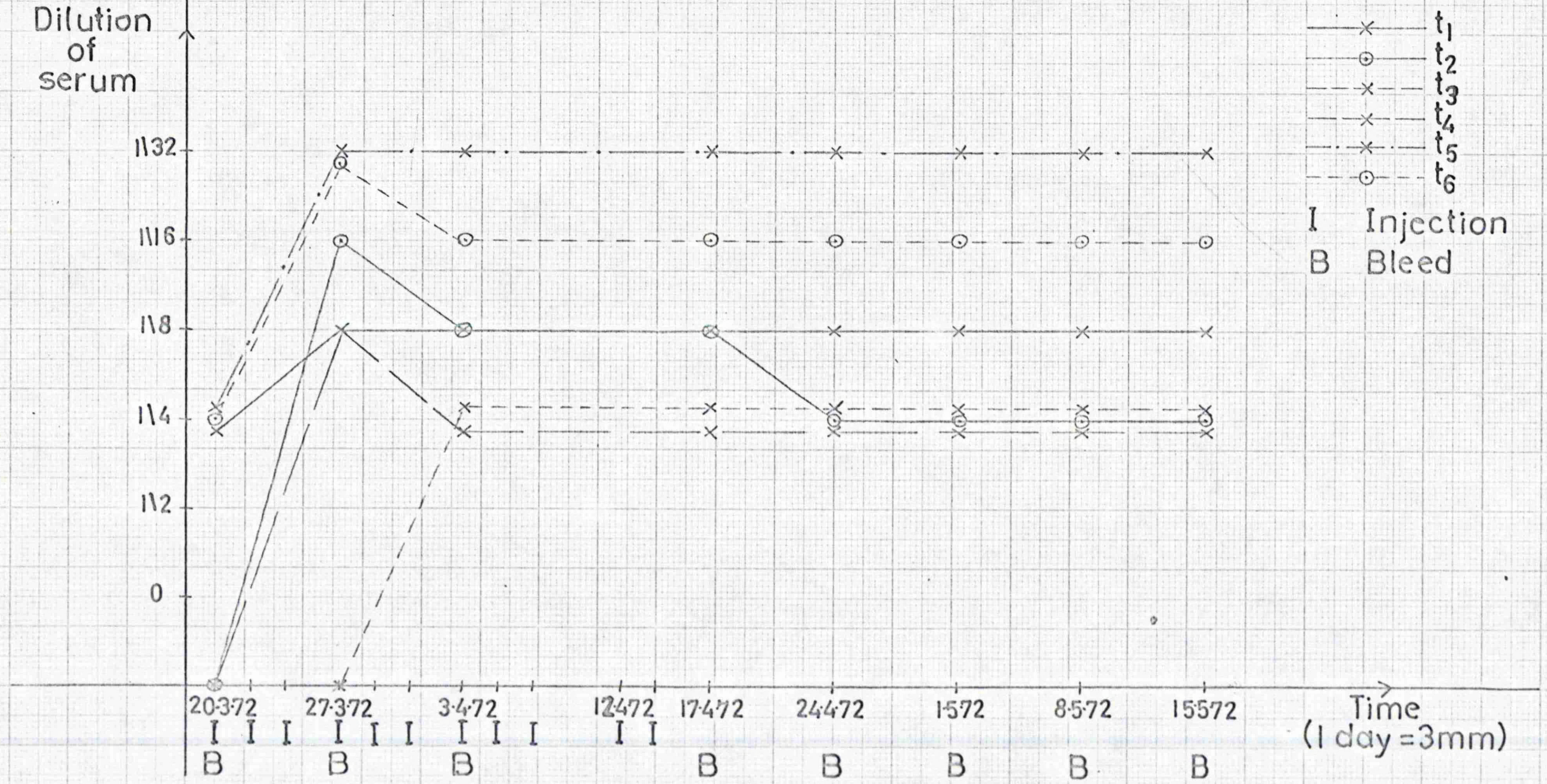
TABLE 3

Comparison of the first course of injections and bleeds given to rabbits 10-13 in 1972. Two breeds of rabbit were involved and all received fresh stigma extract.

Antiserum No.		10	11	12	13
S-allele		S16	S2	S5	S14
Breed of rabbit		C	NZW	NZW	C
No. of injections		24	12	8	12
Type of injections		i/m & s/c	i/m & s/c	i/m & s/c	3 i/v, 9 i/m & s/c
Period of inj. (days)		87	28	26	28
Concn. of inj. (stigmas/ml)		250	250	250	250
Vol. of each inj. (mls)		0.8	0.4	0.8	0.4
Total no. of ab. detected		5	4	5	5
Highest titre reached x days after the first injection	x = 14	1/4	1/4	1/16	1
	x = 28	1/16	1/16	1/32	1/8
	x = 42	1/16	1/8	1/16	1/8
	x = 56	1/24	1/8	1/8	1/8
	x = 80	1/48			
Highest overall titre reached		1/48	1/16	1/32	1/8
S-protein-specific ab.		+	+		
Titre of S-protein-specific antibody		1/48	Unknown		

S-protein antibody. However, that stimulated by the larger injection volumes was of higher titre than that stimulated by the lower injection volumes, but no conclusions can be drawn from this.

GRAPH 14. Titre development of Antiserum 5 after booster course.



CHAPTER 10.

BOOSTER COURSES GIVEN TO RABBITS 5 AND 8.

ANTISERUM 5 continued: S23, $S_{23}S_{23}$, high dominance, kale stigma extract.

This rabbit was previously injected with an S23 stigma extract which had been frozen, thawed and cèntrifuged. At the time the rabbit had been suffering from 'snuffles', and what little blood had been obtained had shown a very poor response of the animal to the injections. Since the rabbit was now healthy it was given a further course of injections of fresh S23 stigma extract.

The rabbit received a total of 1200 stigmas by 7 i/m and 5 s/c injections. Thrice-weekly injections were given over a period of 4 weeks. The rabbit was bled each week except one during the injection schedule and for 4 weeks after injections had ceased.

Dilution Tests.

As can be seen from graph 14, three bands were visible in the pre-injection serum, and the titre of all these had increased by seven days after the start of injections. Two other bands had also appeared in this serum, and a further new band appeared the following week. The initial high level of all but two antibodies was not maintained but titre remained stable during the injection period and for a month after injections had ceased. No further bleeds were taken.

This rabbit showed a similar response to rabbit 6 after a booster injection. The situations were comparable as rabbit 5 had been previously sensitised.

Genotype Comparison Tests.

Absorbed sera.

Samples of all bleeds were absorbed except those taken 20.3.72, 27.3.72 and 8.5.72. The absorbing mixtures contained the following genotypes in different combinations and proportions: 221, S_2S_2; 58/17, $S_{27}S_{27}$; 224, $S_{16}S_{16}$; S21, $S_{21}S_{21}$; A4, $S_{15}S_{15}$ and S14 $S_{14}S_{14}$. All were kales as was the S23.

The serum takeb 3.4.72 was absorbed 3:1 with an extract containing 30 stigmas in 0.1 mls saline. A band pattern similar to that shown ib photograph 91 was produced. Absorption was far from complete, and no specific genotype differences could be seen. The serum was then absorbed 1:1, and no bands at all were visible.

The serum taken 17.4.72 was absorbed in the following combinations:

30 stigmas in 0.1 mls saline, serum absorbed 3:1
37 " " " " " 3:1
30 " " " " " 3:1.5
35 " " " " " 1:1

The first three combinations gave patterns similar to 91, the fourth gave no bands at all.

The 24.4.72 and 1.5.72 sera were absorbed:

30 stigmas in 0.1 mls saline, serum absorbed 3:1
40 " " " " " 3:2

The first gave the 91 pattern, the second gave nothing. Finally, the 15.5.72 serum was absorbed 3:1 with 30 stigmas in 0.1 and the 91 pattern was again produced.

All sera tested gave similar results with these absorbing and comparison genotypes. At some absorption concentration between 30 stigmas in 0.1 mls saline, serum absorbed 3:1.5 and 40 stigmas in 0.1 mls saline, serum absorbed 3:2 the bands visible in 91 were all absorbed out of the serum.

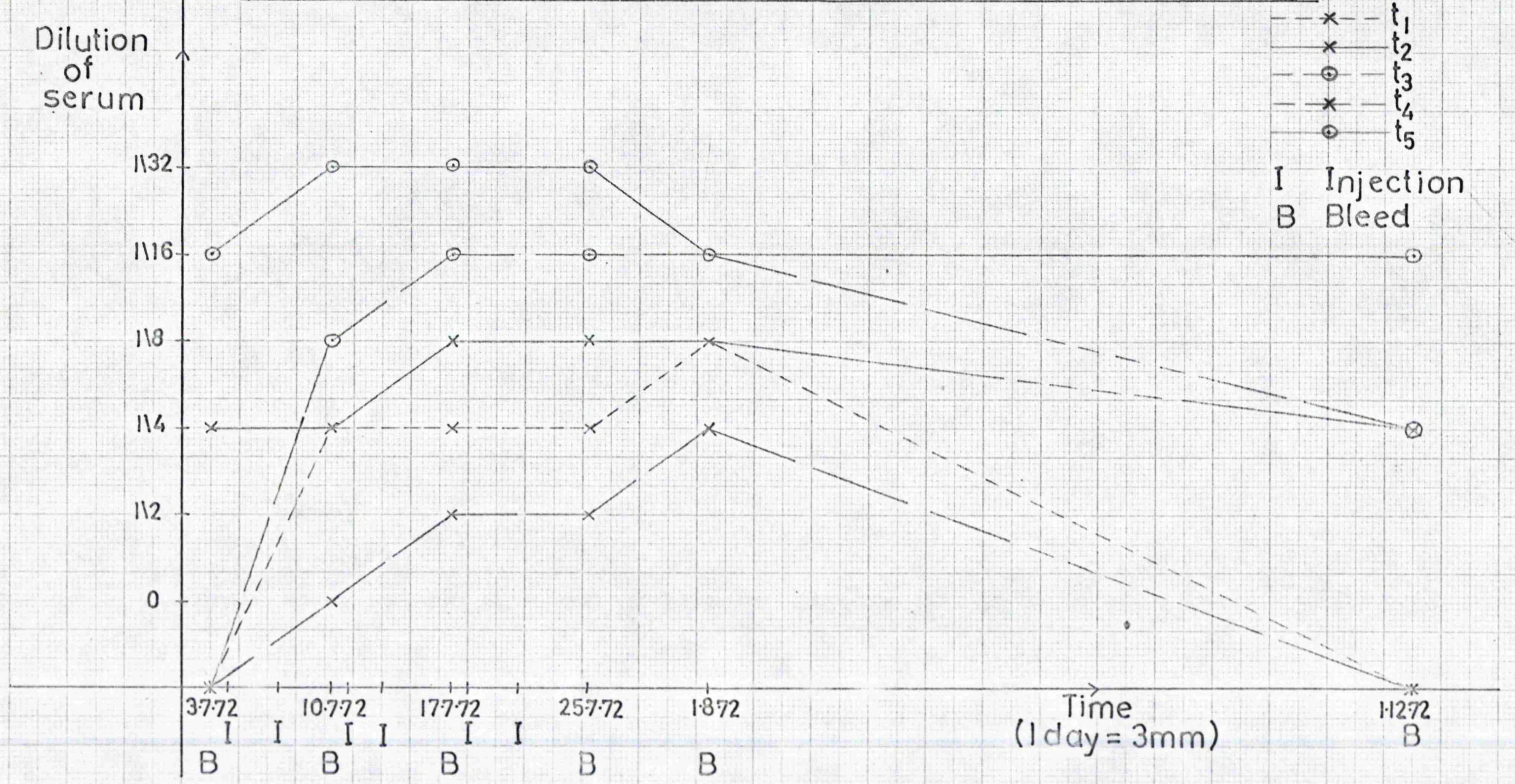
Samples of the serum taken 17.4.72 and 24.4.72 were absorbed and tested as described for antisera 6 and 9, with kales supplied by Dr. Thompson. Absorption was incomplete, but no genotype-specific band was visible.

The sera taken 17.4.72 and 24.4.72 were also used in immunoelectrophoresis tests. The method was as described under antiserum 6. Both sera gave very complex reactions, 11 bands were visible between fresh S23 stigma extract and the 17.4.72 serum as can be seen from photograph 92. The main bands, however, were the same as those produced by antiserum 6 and the same proteins were lost on freezing of the stigma extract. No protein band was specific to the S23 and T7.

Conclusions.

The titre development of this serum followed the same trend as antiserum 6, in the rapid increase in titre and complexity of the serum of the previously sensitised rabbit after further injection. This situation was comparable with booster injections as the rabbit had previously been sensitised, although the last injection had been given seven months previously. As with rabbit 6, an initial dramatic response was shown to the injections, but the rise in titre could not be maintained. However, further injections maintained the titre at a relatively high level for at least a month after injections had ceased. Two antibodies were produced in response to these injections

GRAPH 15. Titre development of Antiserum 8 after booster course.



which were not detected after the first course of injections. The health of the animal probably affected the results obtained after the first course of injections. However, part of the difference may have been attributable to the fact that the rabbit was first injected with extract which had been frozen and thawed.

Antiserum 5 appeared to be even more complex than antiserum 6, although dilution plates did not show this. Immunoelectrophoresis tests showed a maximum of 11 bands against the 17.4.72 serum, whereas a maximum of 9 bands was present against the samples of antiserum 6 tested. As with antiserum 6, immunoelectrophoresis must either be showing bands not visible on the dilution plates or causing separation of the components of what appeared on the dilution plates to be a single band.

The serum contained no S_{23}-specific antibody detectable by any of the methods used as a result of either the first or the second course of injections.

ANTISERUM 8 continued: S45, $S_{45}S_{45}$, intermediate dominance, brussels sprout stigma extract.

This rabbit was given a further course of injections. More serum containing the S_{45}-specific antibody was required and it was hoped that another course of injections would increase the titre of the specific antibody. Twice weekly i/m and s/c injections were given for three weeks, so the rabbit received a total of 1,200 stigmas. Bleeds were taken at weekly intervals during the period of injections.

Dilution Tests.

From graph 15 it can be seen that in the pre-injection bleed only two antibodies remained from the previous course of injections. Three more were produced in response to the injections and the titre of all remained relatively stable. By 1.12.72, over four months after the last injection, three antibodies were still present at detectable levels. Identical bands are represented by the same pattern on graphs 7, 8 and 15.

Genotype Comparison Tests.

Unabsorbed sera.

All sera were tested against a range of genotypes. Patterns as seen in photograph 93 were obtained with all unabsorbed sera. No S-genotype-specific differences could be seen from these plates.

Absorbed sera.

All sera were absorbed with a range of kale and brussels sprout stigmas including S_{23}, S_2, S_5 and S_{15}-containing genotypes. Agarose gels and well pattern 1 were used. Photograph 94 shows the 3.7.72 serum after absorption. This serum was taken before any injections were given, so the bands visible are the result of the injections given previously, the last one being given 13.3.72. No specific band was visible. Photograph 95 shows the 10.7.72 serum absorbed and tested exactly as for the 3.7.72 serum. Here a specific band was present against wells 4, 5, 6 and 7, all of which contained an S_{45}-containing gebotype. Well 6 contained an $S_{23}S_{45}$ heterozygote. Neither of these sera was left overnight to absorb, both were loaded into the gel immediately after mixing of the serum and extract. This resulted in absorption in the gel seen as a halo of precipitate around the antiserum well, thus obscuring partly the S_{45}-specific band in photograph 95. Photograph 96 shows the 17.7.72 serum absorbed. Here the serum was left overnight to absorb and S_{45}-containing genotypes were present in wells 2, 3 and 6.

As in previous tests with antiserum 8 raised in response to boosters, the S_{45}-specific band gave variable results. When visible it was always specific to S_{45}-containing genotypes, but repeatable results were not always obtained and gels were produced showing no S_{45}-specific band.

Conclusions.

If graph 15 is compared with graph 18, it can be seen that only five of the six antibodies were stimulated by this course of injections. This may be due to seasonal changes in the protein complement of the stigmas, or to variation in the response of the rabbit. More likely, however, is the fact that a different S_{45}-containing brussels sprout was used to provide the stigmas for injection. This may have lacked the protein in question or contained a lower, non-antibody-stimulating-concentration. Band t_4 was again visible, and appeared to represent the S_{45}-specific-antibody.

Titre was maintained for at least 3 weeks after maximum titre had been reached after both boosters given to this rabbit. By 3.7.72, almost 4 months after the second booster, only two antibodies were detectable at titres of 1/4 and 1/16. After this course of injections, three antibodies were detectable at a similar length of time after the last injection, two at 1/4 and one at 1/16 titre. Thus, there may be a slight advantage in a full course of injections over boosters given to a previously-sensitised rabbit, as far as titre maintenance is concerned.

Both rabbits 5 and 8 were previously-sensitised animals which were given a further course of injections. Comparison of graphs 15 and 14 shows that tire rise in rabbit 5 was more rapid than in rabbit 8. The initial rapid increase in titre was not shown at all by rabbit 8. This was a similar result to that obtained previously with rabbit 8, when response to boosters was shown to be slower than in rabbit6. Another difference observed bwtween rabbit 8, and rabbits 5 and 6, which gave similar responses, was that increase in the number of antibodies detected after further injections was greater in antisera 5 and 6 than in antiserum 8. Two further antibodies were detected in antiserum 6 after each booster, and two further antibodies were detected in antiserum 5 after further injections. These were not due to differences in the plant material injected as the original plants, and frozen extracts of the stigmas were maintained throughout this work. After the first booster given to rabbit 8, one new antibody was detected, but no further bands appeared after the second booster. No new antibodies were raised after further injections were given 4 months later.

This serum contained an S_{45}-specific antibody, but its presence was not consistent. There are two possible reasons for this. The first is variations in protein content of different batches of stigma extract. The

TABLE 4.

Comparison of titre developments in response to booster injections of fresh extract.

Antiserum number	5	6	6	8	8	8
S-allele	S_{23}	S_{23}	S_{23}	S_{45}	S_{45}	S_{45}
Average concn. of injections (stigmas/ml)	330	500	500	750	330	250
Total number of antibodies detected	6	6	8	6	6	5
No. of previously undetected antibodies	2	3	2	1	0	0 (1 lost)
Highest overall titre reached	1/32	1/32	1/32	1/32	1/32	1/32
Time after booster of max. titre of most ab. (days)	7	6	8	15	10	13
Length of time max. titre of most ab. maintained (days)	14	17	14	49 at least	21 at least	11
S-protein-specific antibody		+	+	+	+	+
Titre of S-protein-specific antibody		1/1	1/2	1/2	1/2	1/4

second is the water content of the gels. As stated previously precautions were taken to ensure that all gels were under the same humidity conditions. All plates of a testwere set up with identical gels made up at the same time and a standard 1 ml of distilled water was used to maintain the humidity of the gel in the petri dish. A lining of filter paper was used to give an even humidity in the petri dish. Whenever possible freshly-prepared gels were used, but otherwise gels were stored in their petri dishes in sealed polythene containers in a constant-temperature incubator. Nevertheless, variations could occur from atmospheric conditions while loading the gels and from different positions in the incubator during development of the plates. With a low antibody titre, a slight difference in concentration caused by dilution in the gel could prevent conditions for precipation. From graph 15, it can be seen that the titre of antibody t_4 was low, reaching a maximum of 1/4.

CONCLUSIONS FROM BOOSTERS GIVEN TO RABBITS 5, 6 AND 8.

Table 4 shows a comparison of the booster injections and courses and the responses to these in rabbits 5, 6 and 8. In all cases, booster injections produced a rapid antibody response. Overall titre was increased to its former maximum level reached after the first course of injections and new antibody specificities were detected. Previously-detected antibodies were often stimulated to higher titre than before.

None of the three rabbits involved had produced an S-protein-specific antibody in response to the first course of injections, but two showed such a positive reaction after their first booster and subsequent booster injections. In both of these cases, the antibody was identified in the dilution plates at a low titre compared with the other stigma-protein antibodies. In antiserum 6, the maximum titre was 1/2, and in antiserum 8 it was 1/4. This suggests that the S-protein-specific antibody is probably stimulated by the first course of injections, but not at a sufficiently high concentration to be detected by the tests employed. The booster injections increase the titre of most antibodies in the serum, including the S-protein-specific antibody, whose titre is raised to a detectable level. This would explain the appearance of other previously-undetected antibodies also. No such antibody was detected in antiserum 5, either because no antibody was produced or because if it was produced its titre was not raised to detectable levels by the booster course.

A comparison of the responses of the three rabbits shows the variation between different individuals. Rabbit 5 produced no detectable S-protein-specific antibody. Rabbits 6 and 8 produced such antibodies but their responses to the boosters was different. Rabbit 6 reached its maximum titre of most antibodies after 6-8 days and maintained it for 14-17 days. Rabbit 8 was slower in the buildup, taking 13-15 days and maintained the titre for much longer periods, at least 7 weeks in one case. Also, booster injections stimulated more previously-undetected antibodies in rabbit 6 than in rabbit 8, only the S-protein-specific antibody being stimulated in the latter. Rabbits 5 and 6 showed a similar response to the boosters, except that rabbit 5 did not produce an S-protein-specific antibody.

Rabbit 8 received two separate boosters and a booster course. The two boosters were considerably different in concentration yet the same response was obtained to both. The booster course involved lower injection concentrations than both previous boosters and resulted in the maintenance of the S-protein-specific antibody. However, one antibody was lost and the highest titre of most antibodies was not maintained for so long as previously. The former can be explained as a different S_{45}-containing brussels sprout was used to provide the stigmas for injection during the booster course. This may have lacked the missing protein. The latter may be a result of the lower injection concentration, but the fact that the titre was maintained for a further 4 months at 1/16 rather than the maximum of 1/32 suggests that the concentration of the injection extract does not greatly affect the response over the range of concentrations used.

The first booster given to rabbit 6 consisted of two injections. From graph 4 it can be seen that the second injection given 6 days after the first had no effect on the response of the rabbit. It neither increased the titre further nor maintained the titre or the complexity of the serum. As a result of this the second booster given to rabbit 6 and the first two boosters given to rabbit 8 both consisted of one injection only. Rabbit 5 was given a course of 12 booster injections and the response is shown in graph 14.

After one week in which it received 4 injections, the titre of most antibodies began to fall and stabilised at a level lower than the initial response to the boosters. Here again, it appeared that further injections did not produce increased stimulation, but they probably served to maintain the titre during and after the injection period. In the case of rabbit 8 titre was maintained in the absence of continued injections, (graphs 8 and 15).

Booster injections appear to increase the probability of obtaining an S-protein-specific response from a rabbit where an initial course of injections has failed. The concentration has little effect on the response and only one booster injection is needed, as further injections do not enhance the initial reaction.

CHAPTER 11.

PROTEIN DETERMINATION, IN VITRO POLLEN GERMINATION AND PAPER CHROMATOGRAPHY.

PROTEIN DETERMINATION.

Protein estimations were started late in 1972, to see if variations from test to test could be correlated with variations in the protein content of the stigma extracts. The method used was that of Lowry et al (1951).

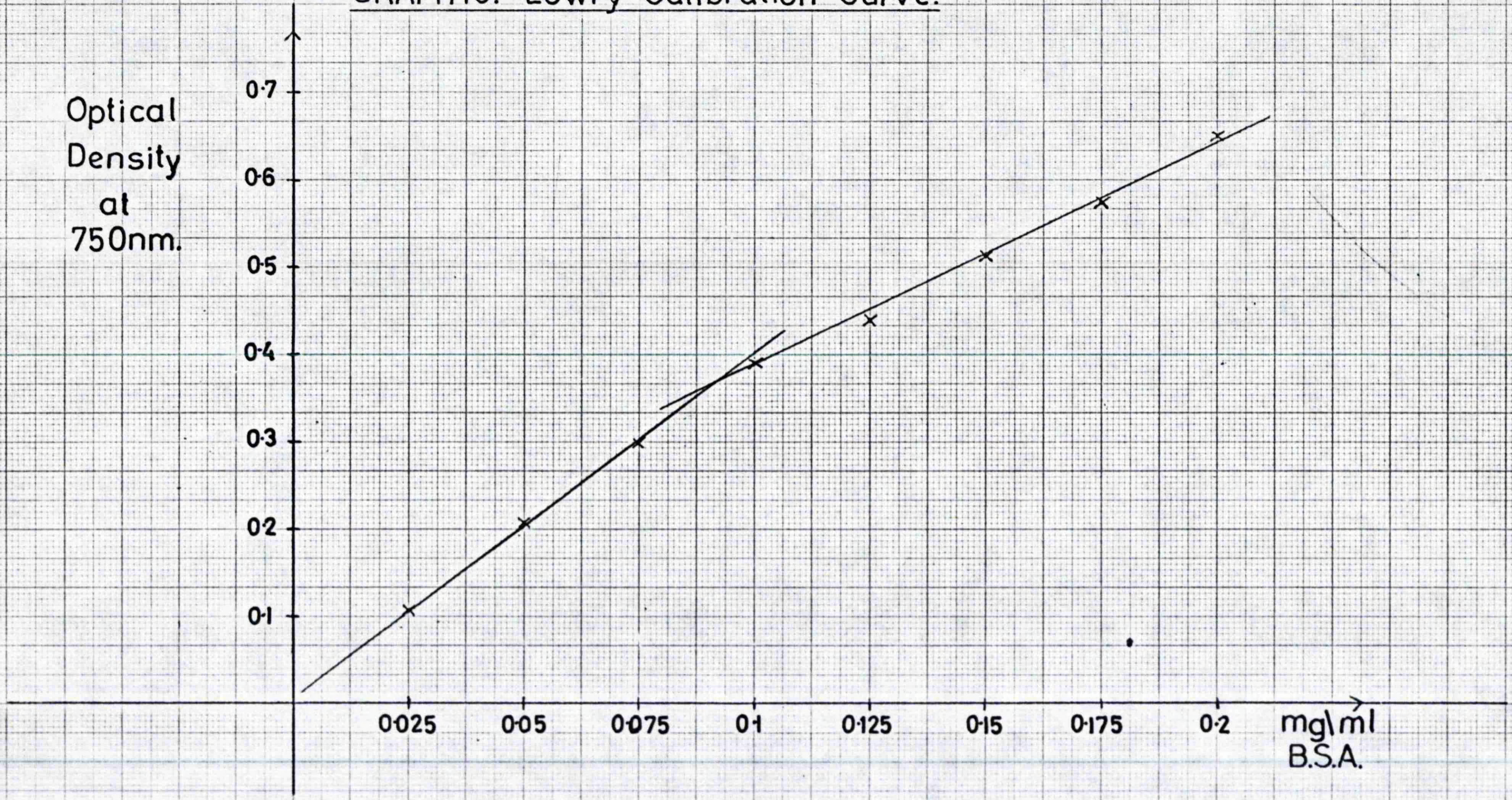
The Lowry reagents were prepared as follows:-

Reagent A - 2% sodium carbonate in 0.1 N sodium hydroxide.
Reagent B - 1% copper sulphate.
Reagent C-2% potassium tartarate.
Reagent D - One part B plus one part C plus 100 parts A, in that order.

Reagents A, B and C were stored, reagent D was made up fresh before each test. All chemicals used were supplied by B.D.H. Ltd. The method was to take 0.5 mls of sample to be tested and add 2.5 mls of reagent D. The mixture was shaken and left for 10 minutes. Folin and Ciocalteau's phenol reagent supplied by B.D.H. Ltd. was diluted in the proportion of five parts reagent to seven parts distilled water. 0.25 mls of the diluted reagent was added to the sample and the mixture thoroughly shaken immediately after mixing. It was then allowed to stand for thirty minutes. The optical density of the sample was read at 750 nm on a Beckman SP500 Spectrophotometer.

A calibration curve was plotted using Bovine Serum Albumin supplied by Calbiochem Ltd. Dilutions were prepared containing between 0 and 0.2 mg/ml. The optical density of each protein concentration was measured

GRAPH 16. Lowry Calibration Curve.



and the curve plotted as in graph 16. The protein concentration of an extract could then be determined from its optical density by reading from this graph.

The standard stigma extract of 25 stigmas in 0.1 mls of buffer was found to have a high protein content. Dilutions of extract of between 1/50 and 1/200 gave readings in the accurate range of the graph between protein contents 0.1 and 0.3 mg/ml. A freshly-prepared extract of a mixture of kale and B.alboglabra stigmas had a protein content of 5.25 mg/ml. After the extract had been stored in the deep freeze for 10 days, thawed and centrifuged, it had a protein content of 3.25 mg/ml. 38% of the protein in the original stigma extract had been lost after freezing.

Conclusions.

A considerable amount of protein was lost when the stigma extracts were stored in the deep freeze and subsequently thawed out for use in gel tests. This protein appeared as a precipitate in the tube and was antigenic protein whose loss could be detected in the gel plates (photograph 10). The precipitated protein did not appear to include the S-protein which was still detectable in gel plates using stigma extract which had been frozen. 38% of the total protein content was lost after freezing and thawing.

The unreliability of the detection of the S-protein, particularly in antiserum 8 did not appear to be due to variations in the protein content of the stigma extract from test to test. Variation in detection occurred from gel to gel in tests set up at the same time with the same batch of stigma extract and gels prepared together. For this reason, and also because of the time involved, the protein content of all extracts was not measured.

IN VITRO POLLEN GERMINATION.

One of the aims of this study was to produce a purified or partly-purified S-protein extract to use as vaccine. This was to enable easier identification of the S-protein antibody than was possible after absorption of all the other antibodies present in the serum. During 1971 and 1972 purification of the stigma extract was not extended beyond freezing of the extract as the only assey for the S-protein was gel diffusion of absorbed serum. As already stated, this was not an efficient method as titre was low and repeatability suspect.

An alternative assey for the presence of S-protein would be to allow it to carry out in vitro its role in the plant, that is, inhibition of germination of pollen containing the same S-allele. *Brassica* pollen germinates very poorly in vitro. Investigations were commenced in 1971 to find a germinating medium which would increase the in vitro germination of *Brassica* pollen so that inhibition of germination could be used as an assey for the S-protein.

This is assuming a number of unproven facts. Although Nasrallah showed the association between the S-protein and the S-allele of a plant by comparing the segregation of the two in the progeny, there is no evidence that the S-protein is the factor exerting the effect of the S-allele. Thus, inhibition of like pollen may not be achieved by the S-protein itself. Another link in the chain between allele and effect may be responsible for inhibition. It is also possible that, assuming inhibitory action, the S-protein cannot function in purified extract, but may need the aid of some other undetected factor. Another assumption is that germination in vitro is a condition directly comparable with germination in vivo. This cannot be so as the nature of the complex interrelationships between pollen tube and stigma are unknown and so cannot be reproduced in vitro.

Pollen tubes were first observed by Amici in 1824 while examining the stigma of Portulaca oleracea. In vitro pollen germination was studied by Schleiden (1849), Van Tieghem (1869) and others. They observed the pollen germination of several plants and concluded that germination varied from species to species and even from variety to variety. Some pollen, such as Pyrus will germinate in distilled water (Knight 1917), but most pollen requires an aqueous solution of sucrose before it will germinate. In 1935, Schmucker discovered that boron as borate was a stimulant to pollen germination and tube growth in many species.

Brassica pollen is trinucleate and, in general, trinucleate pollen is more difficult to germinate in vitro than binucleate pollen (Brewbaker and Majumder 1961). Sisa (1930, 1933) found that cruciferous pollen must be cultivated in a medium containing sucrose and agar. In 1930 he found that a pH of 5 or 6 was unsuitable for germination of Brassica pollen, but that a sucrose-gelatin medium adjusted to a pH of 7.5 with sodium acetate was a suitable basal medium. Schwanitz (1942) found that boron improved the germination of Brassica pollen as had been found for many other genera. Loo and Hwang (1944) germinated pollen of Brassica juncea on a basal medium of 0.6 M sucrose and 5% gelatin. They obtained a germination percentage of between 23 and 29% with pollen tube lengths of between 12 and 14 micra. Manganese sulphate, colchicine and indole-3-acetic acid all increased pollen germination and tube length although the latter two treatments produced abnormal pollen tubes.

In 1967 Minaeva and Gorbaleva found that some flavanoids had a stimulatory effect on pollen germination and tube growth. Quercetin, isorhamnetin, rutin and isoquercetin increased the number of germinating pollen grains per 1000 grains and increased the pollen tube length in all except the last case. The basal medium was a 20% sucrose solution and the pollen was from some Umbelliferae. The effect of flavanoids on the germination of B.oleracea pollen was to be measured and compared with paper chromatographic tests on the flavanoids present in the pollen and stigma. This is described in the next section.

The medium used for the germination of the B.oleracea pollen was as follows:

Oxoid Ionagar No. 2	1.5%
Sucrose	15%
Boric Acid	0.01%
Calcium nitrate	0.03%
Magnesium sulphate	0.02%

Potassium nitrate 0.01%

This was the medium used by Brewbaker and Kwack (1964) except that agar was added and the sucrose concentration was higher. Preliminary tests carried out in 1971 had shown that this medium gave better germination than a simple sucrose, boric acid and agar combination. An agar medium was superior to a liquid or gelatin medium, and 15% sucrose was found to give best germination of a range of sucrose, glucose and maltose concentrations.

The medium was poured onto 3" x 1½" glass microscope slides to a depth of about 3mm. In early tests the pollen was applied to the agar by tapping the anthers of a flower with a needle and allowing the pollen to fall onto the gel. This produced aggregates of pollen on the surface of the agar and it was noticed that only a few of the grains on the outer edge of an aggregate would germinate. Thus, more germination was observed with spaced pollen grains. This was achieved in later tests by tapping the pollen into a watch glass and breaking up the aggregates with a paint brush. The pollen was applied to the gel with the paint brush which deposited spaced grains. Each slide was placed in a petri dish lined with moist filter paper and placed in an incubator at 21°C.

After one hour an average of 6.6% germination was observed by counting the number of germinated and ungerminated pollen grains in three fields of view per slide as observed with a Vickers Stereo microscope. The tubes had an average length of 5.4 times the diameter of the pollen grain. After 16 hours an average of between 13%

and 19% germination was obtained from a range of experiments set up on different dates with pollen from different plants. Germination percentages were constant within one experiment but varied between experiments set up on different dates. Average pollen tube lengths were between 8.2 and 13.5 times the diameter of the pollen grain. No further growth took place after 16 hours, so results were taken after this time.

To facilitate the counting of the pollen grains, photographs were taken of three fields of view of each slide and the percentage of germinated grains and the lengths of the tubes calculated from the photographs. Comparison of the results obtained from observations using the microscope with those from the photographs showed no significant differences between the two methods. Statistical analysis also showed no significant differences in percentage germination and pollen tube length between the slides set up on one date with one batch of pollen, between different fields of view of the slides and between the photographs. As a result of this photographs were used rather than microscope observations for calculation of results. A canon Canola was used to calculate the chi-square values for percentage germination and comparison between treatments. The Wang was used to calculate the analysis of variance between the pollen tube lengths.

The pH of the medium prepared as described was 7.1. This was adjusted with dilute sodium hydroxide or hydrochloric acid to pH 4, 6 and 9 and pollen germination on these media was compared. The maximum % germination was obtained on the medium of pH 7.1 and was 13.42. The maximum tube length was obtained on the medium of pH 6.0 and was 8.23 times the diameter of the pollen grain. There was no significant difference between the % germination on pH 6, 7.1 and 9, but significant differences were obtained between the % germination of pHs 6, 7.1 and 9 and pH 4. As far as pollen tube length was concerned, no significant difference was obtained between pH6 and 7.1, but there were significant differences between pHs 6 and 7.1 and pH9 and between pHs 6 and 7.1 and pH4. The unadjusted pH of 7.1 was used for all further experiments since this gave good results and no advantage was gained by adjusting the pH to 6.

The medium described was compared with a medium containing manganese sulphate at 0.05% concentration, in addition to all the other constituents. Loo and Hwang (1944) had found that the addition of manganese sulphate to the germination medium enhanced the germination of pollen of several herbaceous genera including Brassica juncea. The medium without manganese gave a germination percentage of 13.73% and an average pollen tube length of 12.54. That with manganese gave a germination percentage of 7.51 and an average pollen tube length of 3.67. Significant differences were found for both percentage germination and average pollen tube length, manganese sulphate causing a decrease in both at the concentration used.

Flavonoids which contain a free 5-hydroxyl group react with boric acid in the presence of organic or mineral acids (Wilson 1939). For comparison of the effect of flavanoids on pollen germination, therefore, a medium lacking boric acid had to be used. A basal medium of 15% sucrose and 1.5% agar was used and quercetin and naringenin were incorporated at concentrations of 0.0005%, 0.005% and 0.05%. The control medium gave an average germination % of 10.8 and an average pollen tube length of 5.68. At the 0.0005% level, naringenin had no effect on % germination or on pollen tube length. With the quercetin, however, an average % germination of 21.6 was obtained and an average pollen tube length of 5.39. The difference in % germination was significant at the 0.1% level, the difference in pollen tube length was not significant. When the flavanoids were incorporated at 0.005% no germination was obtained with naringenin which had completely inhibited the pollen. The quercetin gave a germination % and an average pollen tube length which were not significantly different from those of the control. All pollen germination was inhibited by both naringenin and quercetin at the 0.05% level. It appeared from this that naringenin had no stimulatory effect on pollen germination or tube length at the concentrations used, but inhibited at 0.005 and 0.05% Quercetin appeared to stimulate pollen germination at the 0.0005% level, although no increase in tube length was obtained. 0.005% quercetin had no effect on germination or tube length and 0.05% completely inhibited germination. The plates with quercetin were repeated and the same results were obtained, those with naringenin were not repeated.

Conclusions.

The best pH for germination of *B.oleracea* pollen on solid agar medium was found to be 6 or 7. Manganese sulphate at a concentration of 0.05% caused a decrease in % germination and pollen tube length. The flavanoid quercetin stimulated germination at a concentration of 0.0005% and the highest germination of all treatments was obtained with this. There was no effect on pollen tube length. The flavanoid naringenin did not stimulate germination at any concentration used.

PAPER CHROMATOGRAPHY OF THE PHENOLIC COMPONENTS OF POLLEN AND STYLE.

Minaeva and Gorbaleva (1967) stated that the flavanoids quercetin, isorhamnetin, rutin and isoquercetin increased the pollen germination and tube length (except for the last compound) of pollen of some Umbelliferae. The effect of some of these compounds on the germination of *B.oleracea* pollen was to be tested. There is a wide range of phenolic compounds, including the flavanoid pigments, known to occur in angiosperms, (Geissman and Hinreiner 1952, Bate-Smith 1962). A knowledge of those present in the stigma and pollen of *B.oleracea* would facilitate the choice of flavanoid to be incorporated into the germination medium. It could thus be established whether or not the flavanoids present in the stigma serve to stimulate the pollen.

Martin (1969, 1970 a, b & c) and Martin & Telek (1971) have worked with stigmatic exudates and secretions and found that the principal components were phenolic compounds and lipids. Martin (1969) tested ten species from different families and by paper chromatography found that the phenolic compounds occurred as glycosides or esters, and that the greater part of the sugar in the exudates was tied up in phenolic compounds. He suggested that the phenolic compounds may interact with growth substances and account for the specificity of the incompatibility reaction.

Some workers have claimed to have found relationships between sexual reproduction and flavanoids in plants. Kuhn and Low (1949 a, b & c), Moewus (1950 a & b, 1951), and Birch, Donovan & Moewus (1953) put forward evidence which suggested that the reproductive behaviour of *Chlamydomonas eugametos* and *Forsythia* was governed by flavanoids. In the latter case, incompatibility between two varieties of *Forsythia* was claimed to be associated with the presence of rutin in the pollen of one and quercitrin in the other. However, the work on *Forsythia* was repeated by a number of workers but was not corroborated (Esser 1953, Lewis 1954, Esser & Straub 1954, Visser 1956 and Reznik 1957) and the work on *Chlamydomonas eugametos* was also found to be incorrect by Ryan (1956). More recently Namboodiri and Tara (1972) found that different uv absorption profiles were obtained from stigmatic exudates of fertile and sterile species of *Impatiens*. The fertile species had two peaks in the region between 225 and 400 mμ and the sterile species only one. They suggested that the sterile species have a lower stigmatic phenolic content than the fertile species and that the former may be incapable of supporting pollen germination as a result.

Bate-Smith (1962) carried out paper chromatography of leaf extracts and found that members of the Cruciferae contained quercetin, kaempferol, ρ-coumaric acid, sinapic acid and ferulic acid. His survey did not include *Brassica*, but Francois and Chaix (1961) found quercetin-3-rutinoside (rutin) in the seed of B.campestris and Hörhammer et al (1966) found isorhamnetin-3-glucoside in B.napus. Other workers have found robinin, quercetin-3-rhamnosylarabinoside, isorhamnetin, isorhamnetin-3,4-diglucoside, kaempferol-3-rhamnosylarabinoside-7-rhamnoside and kaempferol-3-glucoside in various members of the Cruciferae (Jermstad & Jensen 1951, Stepien & Krug 1965, Maksyntina 1965, Perkin & Hummel 1896, Pacheco 1955, Rahman & Khan 1962, Harborne 1965 and Paris & Charles 1962).

The flavanoid pigments can be divided into a number of different groups (James 1964). All those found in the Cruciferae have belonged to the group known as the flavonols. There are various tests which can be carried out on plant extracts to give an indication of which group or groups of flavonoid are present. These tests were carried out according to Geissman (1955). The extracts ~~tests~~ were obtained by excising 100 stigmas and adding 0.4 mls of solvent which consisted of 95% ethanol plus 1% concentrated hydrochloric acid (Martin 1969). The stigmas were not macerated but were left to extract for 20 minutes. The same was done with pollen from the 100 flowers. The same plant was used throughout and was a 224 kale of S-genotype $S_{16}S_{16}$. Control tests were carried out on the flavanoids quercetin, rutin and naringenin. Quercetin in a flavonol and rutin is a glycoside of quercetin, quercetin-3-rutinoside. These were both obtained from Sigma Chemical Company Limited. Naringenin is a flavanone and was obtained from Koch-Light Laboratories Limited. These controls were dissolved in the extraction medium used for stigmas and pollen to give a concentration of 1 mg in 0.5 mls. The sample extracts and the naringenin were pale yellow in colour, the quercetin and rutin gave slightly darker yellow solutions. The following tests were carried out:

(1) Addition of alkali.

On addition of N sodium hydroxide a deep yellow colour indicates and presence of flavones, flavonols, flavanones, chalcones or xanthones. All the extracts and controls gave a deep yellow colour indicating that one or more of these groups of flavonoids was present.

The addition of cold 0.1 N sodium hydroxide to an extract followed by heating causes a flavanone to isomerise to the corresponding chalcone producing deep yellow to red colours. This did not occur with any of the samples or controls. It is unlikely that a chalcone is present in the stigma or pollen as these impart deep yellow colours to the tissue. Xanthones are of rare occurrence in plants, so the most likely possibilities are flavones or flavonols.

(2) Magnesium/hydrochloric acid test.

Concentrated hydrochloric acid is added dropwise to the extract containing a fragment of magnesium ribbon. The expected colour reactions are as follows:
flavones orange to red, flavonols red to crimson and flavanones crimson to magenta. All the controls gave the expected colour reactions, but neither of the sample extracts showed any change. This was probably because the extracts were not purified at all and waxes or chlorophyll may have affected the result.

(3) Addition of concentrated sulphuric acid.

The expected colour reactions on addition of concentrated sulphuric acid are as follows: flavones and flavonols intensely yellow solutions, flavanones orange to crimson. The quercetin and the rutin both gave intensely yellow solutions but the naringenin showed no change. The stigma extract gave an orange-brown solution and the pollen extract became orange-crimson. This suggests that flavanones were present in the pollen and stigma extracts, although test 1 indicated that this was not so.

(4) Sodium borohydride test.

This was carried out according to the methods of Eigen, Blitz & Gunsberg (1957) and was a specific test for flavanones to check the above result. About 10 mg of sodium borohydride is added to each extract and 1% hydrochloric acid added dropwise until no further release of hydrogen is observed. Concentrated hydrochloric acid is then added and flavanones only give a violet to magenta colour. Of the samples and controls, only naringenin gave a red solution. This indicated that the extracts may not have contained a flavanone, but it must be remembered that in the previous two tests the naringenin gave negative results.

It was concluded from these tests that both the stigma and pollen extracts contained flavanoids which may have been flavones or flavonols. It is possible that a flavanone was present, but there was more evidence against than in favour of this.

Paper chromatography was carried out according to the methods of Bate-Smith (1948), Harborne (1959) and

Martin (1969). The stigma and pollen extracts and the controls were prepared as previously described. The apparatus used was a Shandon T.L.C. Chromatank and Whatman 3 MM paper was used for ascending chromatography. The solvent was n-butanol-acetic acid-water (BAW) in the proportions of 4:1:5. Volumes of 30λ(0.003 ml) were generally applied to the paper, and the extracts were applied unhydrolysed and after acid hydrolysis. This treatment hydrolysed the glycosides so that the aglycone could be identified. An equal volume of 2N hydrochloric acid was added to the extract and the mixture was boiled for 30 minutes. The hydrolysed flavonoids were extracted with 0.1 mls amyl alcohol and this layer was spotted onto the chromatogram. This treatment broke down most flavonoid glycosides. The extracts were concentrated and the paper and spots were dried with a hair dryer. After the solvent front had travelled 15 cm and the paper had been dried, the chromatogram was observed under visible light and under uv light of wavelength 350 mμ. The chromatogram was then subjected to a variety of treatments intended to show the presence or absence of various flavonoids. The treatments used were as follows:

(1) Fuming with ammonia vapour or dipping in 5% aqueous sodium carbonate.

This enhances the colour of some flavanoids especially under uv light. Flavonols and flavones give a particularly distinctive range of colours, flavones fluorescing green and flavonols fluorescing yellow under uv light.

(2) Dipping in 5% ethanolic aluminium chloride.

This causes all flavonoids to fluoresce under uv with yellow or green colours. Chalcones give a characteristic orange fluorescence.

(3) Dipping in a fresh mixture of equal volumes of 1% aqueous ferric chloride and 1% aqueous potassium ferricyanide.

This stains phenolic spots blue.

(4) Dipping in 1% sodium borohydride in isopropanol followed by dipping in concentrated hydrolchloric acid.

A purple or magenta colour indicates the presence of a flavanone.

The ethanolic aluminium chloride revealed no new spots, and nothing was identified by this treatment which was not identified with the ammonia or sodium carbonate.

The ferric chloride and potassium ferricyanide stained most of the detected spots blue, but the results were confused because the unspotted chromatography paper had rows of blue-staining spots which obscured the sample spots.

The sodium borohydride test gave a magenta colour only with the naringenin control. None of the extracts gave a flavanone spot.

In the first runs, 224 only was used as a source of pollen and stigma tissue. Other plants were compared later to see if there were any genotype differences in the chromatogram patterns which might agree with Martin's suggestion that phenolics are involved in the specificity of the incompatibility reaction.

A maximum of 7 spots was detected in the unhydrolysed stigma extracts. Of these, the three with the lowest Rf values were very pale yellow in visible light. Under uv they were deeper yellow and when treated with ammonia or sodium carbonate they became bright yellow and fluoresced under uv. These are the characteristics of flavonols. The Average Rf values of the spots were 0.111, 0.240 and 0.404. In this solvent, glycosides migrate less than aglycones. It was concluded, therefore, that these spots represented flavonol glycosides. The other spots were all of higher Rf and with one exception they appeared purple or blue under uv becoming slightly more green in colour with sodium carbonate. These are the characteristics of isoflavones except that they did not fluoresce yellow with aluminium chloride and uv light. The other spot was yellow-brown and of very high Rf value about 0.95. This was unchanged by all the treatments and its nature is unknown.

In the hydrolysed stigma extracts, all spots were unchanged in appearance and Rf except the two flavonol glycosides of lowest Rf values. The third flavonol glycoside was still visible. On acid hydrolysis most glycosides should be broken down, therefore, this glycoside must be very resistant to acid hydrolysis. A new spot was visible at an average Rf value of 0.78. This spot was yellow under uv light. With ammonia or sodium carbonate solution it became bright yellow and fluoresced under uv light. The Rf of this spot was a little lower than that of the quercetin control at 0.81. However, it was concluded that this was, in fact, quercetin, as the rutin control, on hydrolysis, gave quercetin with an Rf value of 0.765. The acid hydrolysis had caused the flavonol glycosides of Rf 0.111 and 0.240 to break down to quercetin and unknown sugars. The identity of these glycosides was not established as this would require further chromatography in different solvents, identification of the sugars and spectral analysis.

A maximum of 5 spots was detected in the pollen, but these were not so clear as those in the stigma. No flavonol glycoside was consistently detected in the unhydrolysed extract, but one may have been present at the rather high average Rf value of 0.5. In the hydrolysed extracts, however, there appeared, in most cases, to be an aglycone of average Rf 0.79. Here again, therefore, glycosides of quercetin were probably present in the pollen, although it was not ascertained as to whether or not these were identical with those in the stigma.

Four different genotypes were compared with the 224 kale on stigma extracts only. These were S23 an $S_{23}S_{23}$ kale, BB1 an $S_{45}S_2$ brussels sprout, KB2 an $S_{16}S_2$ kale/brussels sprout hybrid and KK1 an $S_{23}S_{16}$ kale. The brussels sprout parent of KB2 was the same as the S_2-containing parent of BB1 and the kale parent was 224. The S_{16}-containing parent of KK1 was 224 and the S_{23}-containing parent was the S23. Thus four different S-alleles were involved in different combinations. However, no genotype differences were detected in either the unhydrolysed or the hydrolysed stigma extracts.

Conclusions.

The stigma extracts of B.oleracea contains at least three glycosides of quercetin, at least one may be present in the pollen extract. No genotype differences have been detected between the five plants compared.

Harborne (1967) stated that 3, 7-glycosides of quercetin fluoresce in uv with ammonia, whereas 3-glycosides do not, and that a flavonol glycoside with a sugar in the 7-position is more slowly hydrolysed than one with a sugar in the 3-position. Thus 3, 7-glycosides are more likely to be present in the stigma. The glycoside with the Rf of 0.111 must have more than one sugar attached as increasing glycosylation results in lower Rf values in BAW.

<u>Conclusions from Protein Determination, In Vitro Pollen Germination and Paper Chromatography.</u>

The protein determinations carried out on the stigma extracts showed that after freezing and thawing 38% of the protein content was lost from solution as a precipitate. This loss of protein could be seen from the gel plates (photograph 10) but otherwise its nature was unknown. However, the lost protein did not appear to include the S-protein as can be seen from a comparison of photographs 11 and 12.

<u>B.oleracea</u> pollen would germinate on a basal medium of 15% sucrose and 1.5% agar to give a maximum of 10.8% germinated grains with an average pollen tube length of 5.68 times the diameter of the pollen grain. Addition of 0.01% boric acid and various salts increased germination to a maximum of 19% with a maximum average pollen tube length of 13.5 An increased germination % of 21.6 was obtained with the basal medium plus 0.0005% quercetin, but the average tube length was not increased over that obtained on the basal medium. The other

flavanoid whose effect on pollen germination was tested was naringenin, but no increase in pollen germination or pollen tube length was observed at any concentration.

Paper chromatography of stigma and pollen extracts showed that in the stigma there were at least three and in the pollen at least one glycoside of quercetin. The free aglycone was not found in either tissue. As stated previously, 0.0005% quercetin had a stimulatory effect on pollen germination. It is possible that the quercetin glycosides in the stigma may stimulate the pollen and that the free quercetin and sucrose in the germination medium may fulfil this role to a certain extent. Naringenin was not found in the stigma or pollen tissue and did not stimulate the pollen. The quercetin was not involved in the incompatibility reaction as no difference in the quercetin glycosides was observed between the stigmas of five different genotypes containing four different S-alleles. The quercetin affected only the pollen germination, no increase in pollen tube length was observed. Thus, some evidence was obtained in support of Martin's suggestion that phenolics may be involved in controlling pollen germination (Martin 1969). There was no evidence, however, to suggest that phenolics were involved in intraspecific incompatibility in <u>B.oleracea</u>.

CHAPTER 12.

CONCLUSIONS AND DISCUSSIONS.

The main conclusion to be drawn from this study is that serological methods cannot be used as a routine S-allele diagnostic test for B.oleracea without further refinement. As it stands, the technique would be far too expensive and unreliable.

The main problem was the low antigenicity of the S-protein. Eleven rabbits were injected with stigma extract in 1971 and 1972, only four of which produced an S-protein-antibody. Moreover, two of these were stimulated in response to booster injections after the first course of injections had ceased. These results are in agreement with those of Nasrallah (1965). He injected 24 rabbits, 12 with stigma and 12 with pollen extract. None of the rabbits injected with pollen extract produced an S-protein antibody and only three of those injected with stigma extract gave such an antibody. Of these only one was described as "strong" and the other two were "weak". (Nasrallah 1967a), Mäkinen & Lewis (1962) achieved similarly poor results with Oenothera organensis pollen extract. Eleven rabbits were injected, three of which produced no response to any protein in the pollen extract. Of the remaining eight antisera only three were described as "very weak". S-antibodies were builtup to two S-genotypes only. The maximum number of precipitation lines in any serum was three.

Another result of the low antigenicity of the S-protein was the low titre of its antibody in the sera of the rabbits which did respond to it. As detected by dilution tests, the S_{45}-antibody had a maximum titre of 1/4 and the S_{25}-antibody of 1/2. The S_{16}-antibody reached a much higher titre of 1/48 but the situation was confused by reduced specificity of the S_{16}-antibody. Results were clearest when its titre was 1/16. The S_2-antibody was never identified on the dilution plates but its titre must have been quite low because of its faint response in gel plates and also because the highest titre reached by any antibody was 1/16 and this was detected on one date only. It must be remembered that dilution plates were carried out on unabsorbed serum. After absorption the titre was reduced by dilution and using well pattern 6 the titre of the S_{16}-antibody was 1/4 rather than the 1/16 detected by dilution plates. Evidently the different well spacings and the smaller antigen wells réduce the detectable titre still further.

Before the technique could be used as a routine for S-allele diagnosis, some method of increasing the antigenicity of the S-protein would have to be found. Purification may be of advantage here as antibodies have been detected to at least eight components of the stigma extract other than the S-protein. If these were removed, then the rabbits antibody-producing system may be channelled exclusively into production of S-antibody resulting in a higher rate of success and a higher titre of the specific antibody. Extraction of the S-protein by diffusion from the unmacerated stigmas may provide the first step in purification as it has been shown using antiserum 10 that the S_{16}-protein diffuses readily from the stigmas.

Purification of the S-protein would produce antibodies exclusively to the S-protein. This would obviate the need for absorption and so would avoid the resulting dilution of the serum.

It has been shown that rabbits vary in their response to the S-protein. Rabbit 10 rapidly produced an antibody of relatively high titre, rabbit 11 produced a low titre antibody after a short period of injections and rabbits 6 and 8 needed booster injections before low-titre S-antibodies were stimulated to detectable levels. The other rabbits injected produced no S-antibody at all. It has been shown that increased resolution of the S_{23}-antibody was obtained by concentration of the serum using Lyphogel, the repeatability of results from low titre antibody could probably be increased by this means. Lyphogel could also be useful in concentrating antibody of higher titre, so that diagnostic gel tests could be carried out with the minimum of stigma tissue, thus making the test as easy as possible. Research would have to be carried out on the minimum number of stigmas needed to prepare sufficient vaccine to stimulate a specific antibody response as, at present, a great deal of time and labour is needed to inject one rabbit as a single injection extract takes between 1 and 5 hours to prepare. This would have to be reduced or the cost involved in raising antisera to over 40 different alleles would be excessive.

From a comparison of the results obtained from dilution plates, genotype-comparison plates and immunoelectrophoresis it can be seen that all had their

TABLE 5

Comparison of all antisera raised in 1971 and 1972

Antiserum number	State of inj. extract	Average concn. of extract	Highest overall titre	Total no.ab. detected	S-protein-specific ab.
1	Frozen	600	1/8	2	
2	Fresh	1000	1/32	6	
3	Frozen	1000	1/32	5	
5 1st course	Frozen	550	1/8	4	
Booster course	Fresh	250	1/32	6	
6 1st course	Fresh	550	1/32	3	
1st booster course	Fresh	500	1/32	6	+
2nd booster course	Fresh	500	1/32	8	+
8 1st course	Fresh	700	1/32	5	
1st booster course	Fresh	750	1/32	6	+
2nd booster course	Fresh	330	1/32	6	+
3rd booster course	Fresh	250	1/32	5	+
9	Formalin treated	650	1/8	4	
10	Fresh	250	1/48	5	+
11	Fresh	250	1/16	4	+
12	Fresh	250	1/32	5	
13	Fresh	250	1/8	5	
7 (pollen)	Fresh	Pollen from 1000 fls/ml	1/16	6	

limitations in assessing the sera. Immunoelectrophoresis revealed more antibodies in the sera but did not show the antibody in antiserum 6 specific to the S23 and T7 genotypes. All three methods are needed for the best assessment of a serum, but the conditions in the immunoelectrophoresis experiments were not suitable for the precipitation of all antigen-antibody complexes.

During this work it was shown that the S_{SD7} allele was identical with the S_{45}-allele of Dr. Thompson and that S_{45} is dominant to S_2 in the pollen. A test which was not repeated showed that stigmas homozygous for the S_{45}-allele contained more S_{45}-protein than stigmas heterozygous for the allele. This was also indicated by results with antisera 6 and 11 although experiments were never set up to confirm it.

Table 5 shows a comparison of all antisera raised in 1971 and 1972. Of the eight sera raised in 1971, none contained an S-antibody. Three sera were raised to stigma extract which had been frozen, thawed and centrifuged and one to formalin-treated extract. These four sera gave poor results. Compared with those raised to freshly-prepared stigma extract, lower overall titre and fewer detectable antibodies were produced. Stigma extract which had been frozen still gave satisfactory results in the gel plates and the S-protein was not lost. However, such extract was less antigenic in the rabbits. The protein must have been altered in such a way that it was still capable of reacting with its antibody but the rabbit's defence system either did not recognise the altered protein to the same extent or was capable of destroying it more easily. Antiserum 7 was raised to pollen extract. No S-antibody was raised, but the pollen proteins appeared to be more antigenic than the stigma proteins. They gave much clearer precipitin reactions, even with stigma antiserum and their antibody titres were maintained for longer periods. This was also found by Nasrallah (1967a) who was unable to find an S-antibody in any of the 12 sera he raised to pollen extract.

The results from these first eight rabbits showed that the length of the injection course, the inclusion of i/v injections and the concentration of the injection extract over the range used had no significant effect on the response of the rabbits. All variation, apart from that described, could be accounted for by the variation in response from rabbit to rabbit.

Booster injections were given to rabbits 5, 6 and 8. A rapid titre increase was obtained in response to these, but this was not maintained except in the case of antiserum 8. Booster injections resulted in the detection of an S-antibody in antisera 6 and 8, and other new specificities were detected also, especially in the case of antiserum 6. In both cases lower injection concentrations were used than for the first course of injections and the variations in the response of different rabbits was also shown. Rabbit 8 showed a slower titre buildup than rabbit 6 but maintained its titre for longer. Also, fewer previously-undetected antibodies appeared in rabbit 8 serum after the boosters than in rabbit 6 serum. Rabbit 5 produced no detectable S-antibody but otherwise its reaction was similar to that of rabbit 6. One injection only appeared to be sufficient to stimulate the full booster reaction, further injections had no apparent effect.

Four rabbits were injected in 1972 and all received relatively dilute, freshly-prepared injection extracts of 250 stigmas per ml. Two of these rabbits produced an S-antibody. Thus, boosters and dilute injection extracts were the only treatments which stimulated an S-antibody. The inclusion of i/v injections in the course given to rabbit 13 produced a poorer response than the courses of equal length of i/m and s/c injections given to the other rabbits. The variation within the N.Z.W. breed of rabbit appeared to be as great as that within the Californian breed, an S-antibody was produced by one of each breed. The long course of injections given to rabbit 10 was of no advantage as titre was hardly raised and the S-antibody appeared to become less specific after two months of injections. This led to confused results in the gel plates as an apparent cross-reaction occurred with S14 stigma extract. From the short gap in the injections given to rabbit 10 between 13.4.72 and 24.4.72 it was found that there was a lag period of 10 days between the cessation of injections and the fall in titre and a lag of 8 days between the recommencement of injections and the increase in titre.

Since no serum had an overall titre of higher than 1/48 as detected by dilution plates, it was concluded that the stigma proteins of B.oleracea are not very antigenic in rabbits. In addition, the bands were never very clear. Some pollen proteins gave clearer bands than the stigma proteins, although their titres were not higher. Fulton (1967) when working with labile plant viruses concluded that the reason for the poor antigenic response to these viruses was due to their small size compared with the majority of plant viruses. Nasrallah (1967b) found that the S-protein had a molecular weight of 150,000. This would give a small particle, far smaller than most plant viruses and may explain the poor results.

In general it appeared that the more dilute injection extracts were most likely to stimulate an S-antibody. None of the sera raised to extracts more concentrated than 250 stigmas/ml produced an S-antibody except by booster injections. However, Nasrallah when at S.H.R.I. prepared his extracts for injection as concentrated as possible. Concentrated extracts did not produce higher overall titres than more dilute extracts.

Five rabbits received i/v injections and four were bled after these had been given. In none of the cases tested was any response detected in the serum. Titre was detected after a similar course of i/m injections as shown by antiserum 10. Nasrallah (1967b) stated that one of his S-antibody-containing sera was produced solely in response to i/v injections. However, the replacement of the first week of i/m injections with i/v injections in the case of rabbit 13 produced a lower antibody response than in the other three rabbits which received a full course of i/m and s/c injections.

Of the four S-antibodies, two were raised to alleles of intermediate dominance and two to alleles of high dominance. Two low-dominance alleles were tested, S_{15} and S_5 but neither stimulated the production of an S-antibody. However, the S_{15}-serum was raised to extract which had been frozen, so a poor antibody response was to be expected. The rabbit injected with the S_5-containing extract reacted very rapidly to the injections and a titre of 1/32 was reached only 28 days after the first injection. Thus, only the second case would have been expected to produce an S-antibody. Of the three sera raised by Dr. Nasrallah, two have been identified with Dr. Thompson's alleles (Nasrallah, Wallace & Savo 1972). The S_1-allele was Thompson's S_2 and the S_2-allele was S_{14}. The former is of intermediate dominance and the latter of high dominance. The S_{14} gave the 'strong' antiserum. The third S-antibody and the S-allele which did not stimulate an S-antibody have not been identified. Thus, of the few alleles tested, only those of high dominance have stimulated a 'strong' S-antibody, S_{14} as produced by Nasrallah and S_{16} as produced in this study. Of the other S-antibodies whose S-allele was identified, all were raised to alleles of intermediate or high dominance. It cannot be concluded that such alleles are more antigenic than those of low dominance, but there are indications that this may be so.

Tests with antiserum 10 showed that the S_{16}-protein was present in the stigmas but not in the pollen. If a substance involved in the incompatibility reaction exists in the pollen, then it would be expected to diffuse as freely as the stigma protein. The fact that it does not react with the S_{16}-antibody suggests that the incompatibility substance is not identical in the pollen and stigma of B.oleracea as found by Linskens (1960) with Petunia.

The work with gelatin caps and antiserum 10 also suggests something regarding the mechanism of the S-allele action. The diffusion of the S_{16}-protein into the gelatin caps on the stigmas did not prevent the germination of the plant's own pollen. This suggests that either the S-protein does not inhibit germination of the pollen, or that it cannot function extracellularly, or that it cannot function when diluted.

As a result of the protein estimations it was found that between 35 and 40% of the protein content of a stigma extract was lost on freezing. Results of immunoelectrophoresis experiments showed that this protein lost on freezing was all anode-migrating.

Germination of B.oleracea pollen occurred on a basal medium of 15% sucrose and 1.5% agar, 10.8% germination took place and the tubes had an average length of 5.68 times the diameter of the pollen grain. The highest pollen tube length of 13.5 times the diameter of the pollen grain was obtained on the above medium with the addition of 0.01% boric acid and various salts. The % germination was increased to a maximum of 19% and the optimum pH was 6 or 7. The highest % germination was obtained when 0.0005% of the flavanoid quercetin was incorporated into the basal medium. 21.6% germinated grains occurred but the pollen tube length was not increased over that obtained on the basal medium.

Naringenin, which is also a flavanoid did not stimulate either pollen germination or tube length.

Paper chromatography of stigma and pollen extracts showed that quercetin, but not naringenin, was a constituent of the stigmas of all the plants tested and that it was probably found in the pollen also. The quercetin was not found as the aglycone, but as glycosides, at least three being present in the stigma and at least one in the pollen. It is possible that the quercetin glycosides in the stigma may stimulate the germination of the pollen. It is not involved in the stigma incompatibility reaction, however, as no difference in the glycosides of quercetin was observed between the stigmas of five different genotypes containing four different S-alleles.

The formation of new S-allele specificities is generally thought to be brought about either by true point mutations or by intracistronic crossing-over (Pandey 1956, Lundquist 1965), the recent evidence on the occurrence of new specificities during inbreeding possibly pointing more to the latter cause. Either mechanism suggests a degree of relationship between all the S-alleles at a locus and particularly between an allele and the one derived from it. Structural relationship would suggest antigenic similarity and unless a large number of mutations is needed before a new specificity can arise, it seems unlikely that the S-proteins are sufficiently different for each to stimulate a totally different antibody. This could explain the small proportion of sera containing an S-antibody as the only method of detection of the S-antibody is by gel test after absorption of the serum. If the S-proteins stimulate the same or very similar antibodies, then absorption would remove the specific antibody. Thus the only S-specificities to be detected would be those only very distantly related to the S-proteins in the plants used for absorption.

Some evidence of possible relationships between S-proteins was obtained during the course of this work. It was noticed that in all cases, but particularly with antiserum 10, that the titre of the S-antibody after absorption of the serum was far lower than that before absorption and that the band also appeared less distinct (graph 10, photographs 70 and 76). This could be because the band detected before absorption in fact consisted of more than one antibody specificity, all unrelated to the S-locus except for the S-antibody itself, and there is some evidence from immunoelectrophoresis that this may be so. However, it could also be due to relatedness between S-alleles. If, for example, a particular S-allele has a number of antigenic determinants, all of which stimulate antibody specificities, then absorption with a related S-protein might remove some, but not all, of the antibody components of this complex, thus reducing its overall titre and the distinct appearance of the resulting band. The cross-reaction with the S14 stigma extract which arose during the later stages of the injection schedule of rabbit 10 (photographs 72, 73 and 74) is also evidence in this direction. The cross-reaction never appeared as a band separate from the S_{16}-band in the gel plates and it is well known that antibodies become less specific after prolonged injections. It is possible that a new specificity was stimulated to the S-protein molecule either to a different determinant, or to a combination of determinants, and that this particular specificity was shared by the S_{14}-protein. However, in the absence of a specific S_{14}-antiserum, there was no evidence that this cross-reacting protein was associated with the S_{14}-protein.

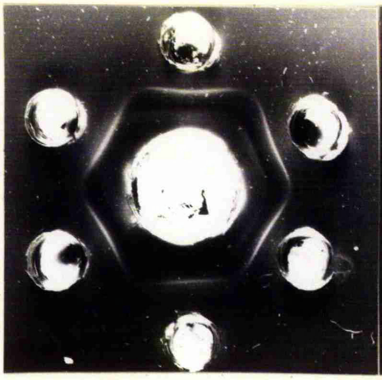
Antiserum 11, raised to the S_2-allele, provides the best evidence for the possible non-discrete nature of the S-proteins. When the serum was absorbed with a mixture of brussels sprout, cabbage and kale stigmas involving the S_{15}, S_{45}, S_2 and S_{23} alleles, an S_2-antibody could be detected which showed a reaction of identity between stigma extracts from a brussels sprout (7872) and a kale (221), both homozygous for the S_2-allele. However, when an S_5S_5 brussels sprout was included in the absorption mixture, a spur was produced by the 221 which extended beyond the 7872-band (photographs 87 and 88). This suggests that the altered absorption mixture had removed a specificity from the 7872 which was not removed from the 221 and indicates that the background in which the S-allele occurs may affect its structural specificity. Possibly the S_5-protein in a brussels sprout background shares a specificity with the S_2-protein in a brussels sprout but not in a kale background. Alternatively, the specificity may be attributable to other factors such as modifier genes affecting the action of the S-protein. The S_5S_5 brussels sprout was partially self-compatible, regularly allowing 15-20 pollen tubes per self-pollination. The 7872 rarely allowed more than 5 pollen tubes per pollination, but the 221 was almost completely self incompatible, occasionally allowing one or two pollen tubes to grow down the style. A difference such as this, possibly governed by modifier genes which affect the S-protein in a structural, antigenically-detectable manner, might explain this specificity common to the 221 but not to the 7872.

Thus, errors in interpretation might arise due to the incompatibility genotypes used for absorption and to the genetic backgrounds of the plants. All these variables would have to be investigated and elucidated before the method could give reliable results, and even then, unless an alternative could be found to absorption, S-allele identification would depend upon what may be very small differences between closely-related proteins which may not always be serologically distinguishable.

Although this work did not investigate the actual mechanism of incompatibility, it would appear that there are antigenic proteins in the stigmas of B.oleracea plants which are associated with the S-allele status; one protein in a homozygote and two in a plant heterozygous at the S-locus. There is no indication as to the function of the protein, whether it is the factor which effects the inhibition of like pollen or whether it is some link in the chain between gene and gene action. However, it has been shown that this protein diffuses readily onto the surface of the stigma, and this fact lends some support to Ockendon's (1972) theory that incompatibility is governed by some specific substance whose site of action is the stigma surface.

From the sera tested so far, it would seem that the most successful injection schedule is one of twice-weekly i/m and s/c injections of fresh extract of about 200 stigmas in 0.8 mls of buffer. An injection schedule of between 6 weeks and 2 months should allow adequate titre response of any rabbit. Injections should not be continued beyond this time as cross-reactions or new specificities may occur as with antiserum 10. These serve only to confuse the reaction. Bleeds can be taken from a month after the start of injections until a month after the cessation of ihjection, preferably at weekly intervals. If more serum is needed at a later date, a single booster should be sufficient to raise the titre of all antibodies to their highest former level. For any particular S-allele to which an antibody is desired, at least two rabbits need to be injected. A possible improvement on this schedule would be to sensitise the rabbit about a month before the injection schedule proper. From the work carried out it would appear that i/m injections are better than the usual i/v injections for sensitisation. A sensitisation of a few i/m injections has not been tested in this work, but after a full course of injections, rabbit 5 remained sensitised for seven months at least.

Possible improvements to this schedule have already been described. They include purification and possible concentration of the S-protein, concentration of resulting sera with low S-antibody titres and research into the minimum number of stigmas needed to stimulate an S-protein-specific response. As it stands, the technique is unsuitable for routine S-allele diagnosis in *B.oleracea.*



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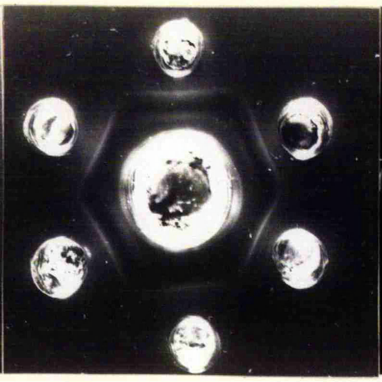

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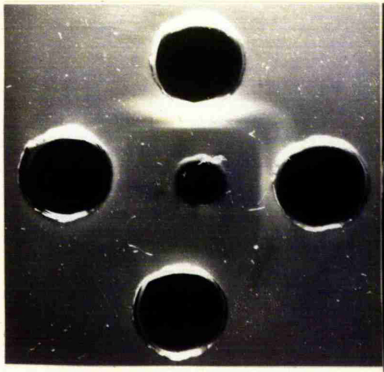

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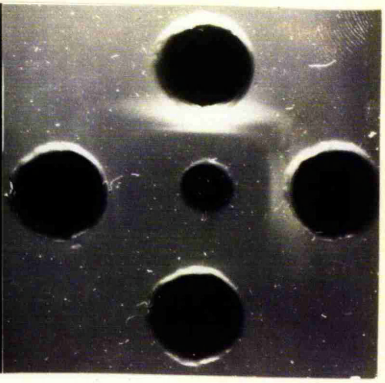

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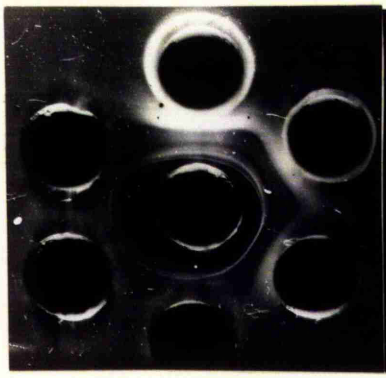

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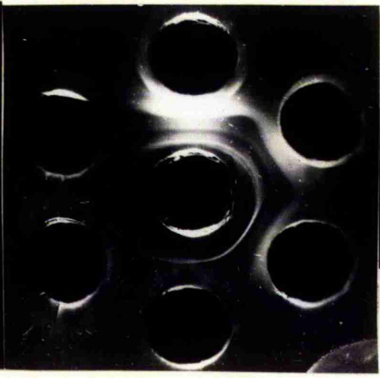

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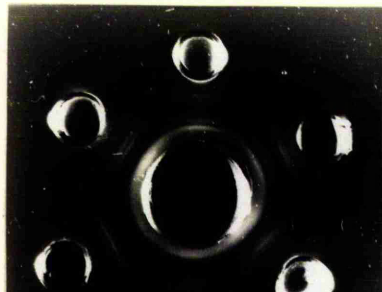

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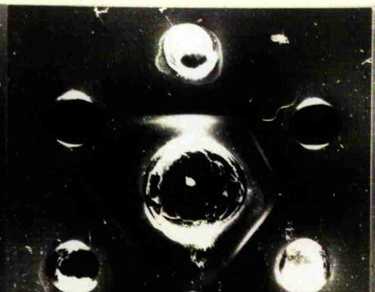

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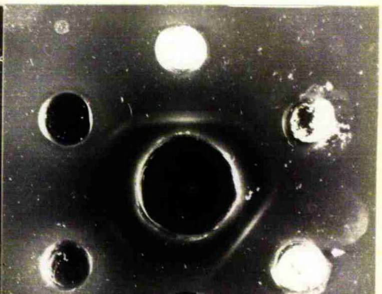

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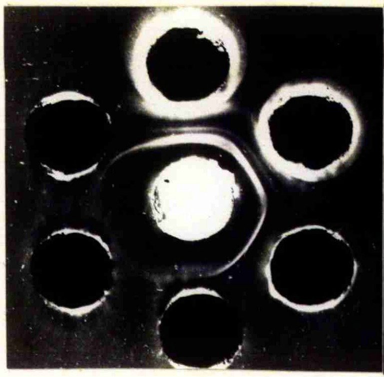

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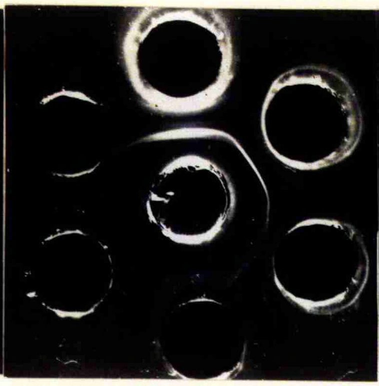

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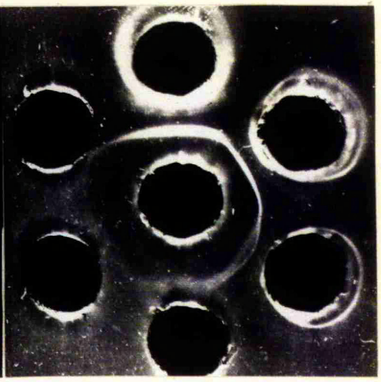

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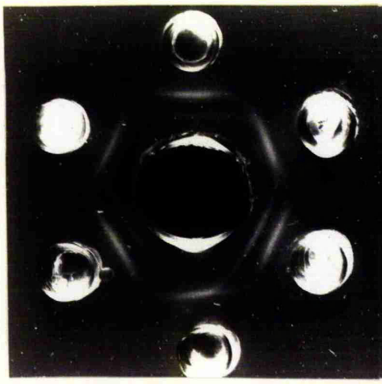

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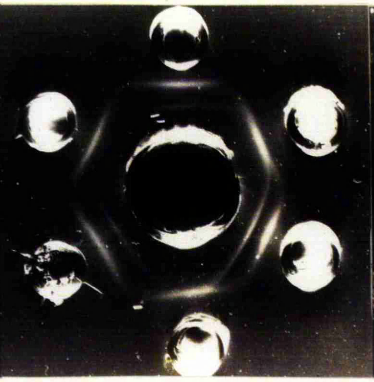

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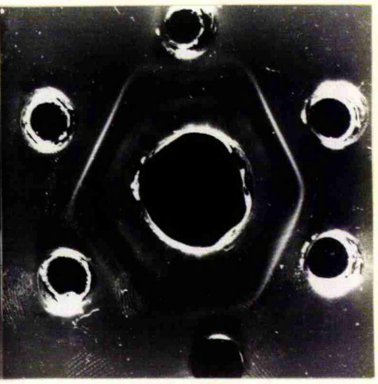

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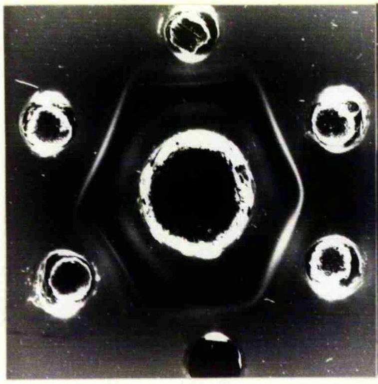

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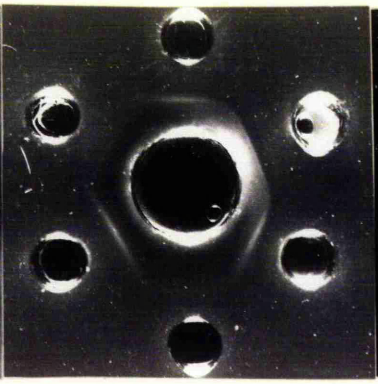

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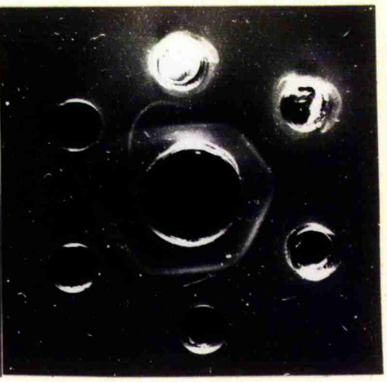

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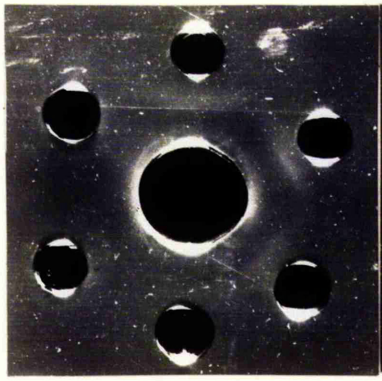

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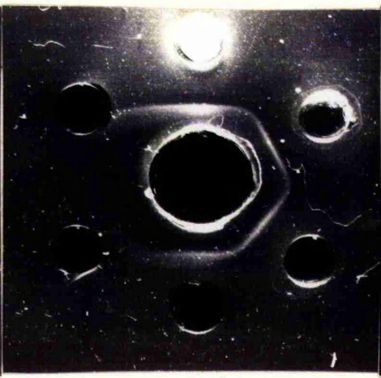

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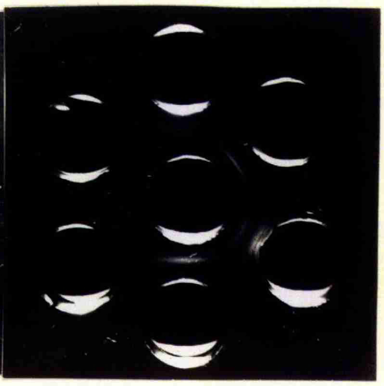

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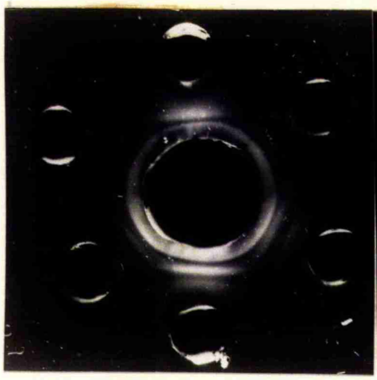

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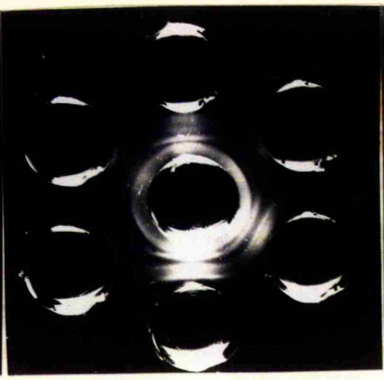

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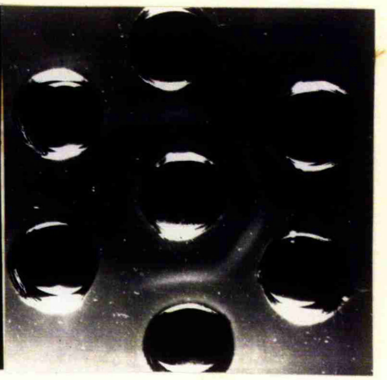

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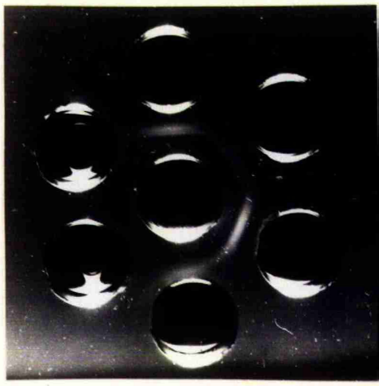

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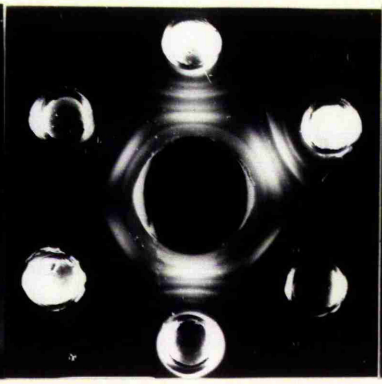

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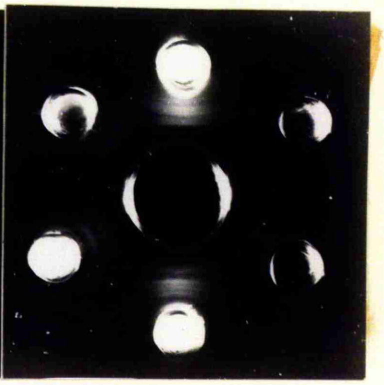

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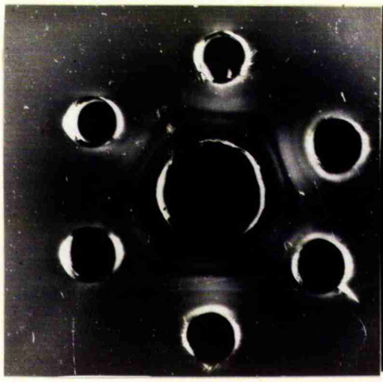

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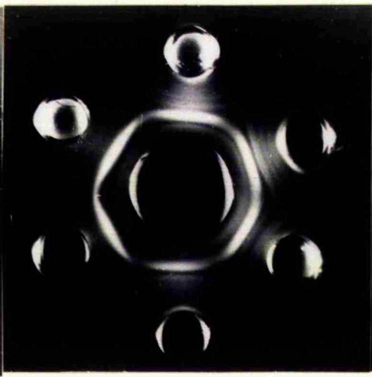

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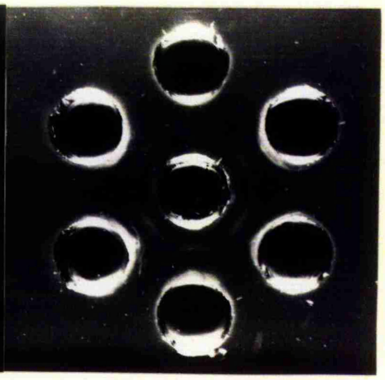

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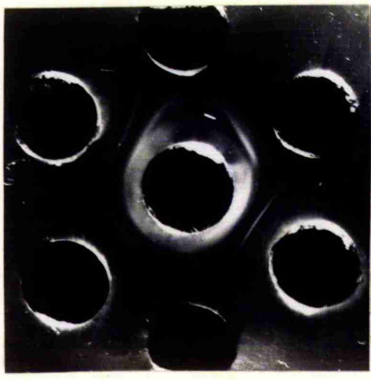

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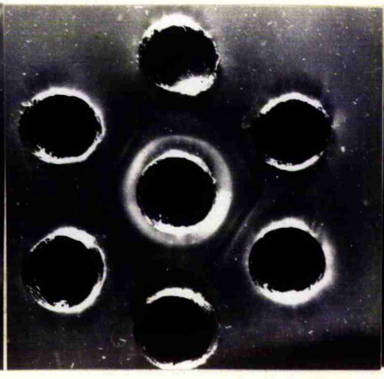

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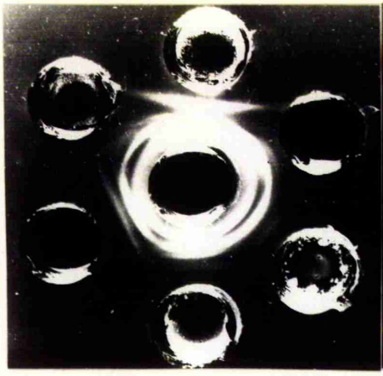

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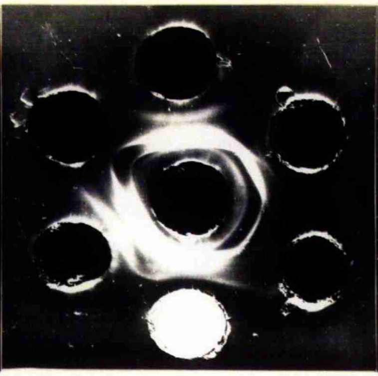

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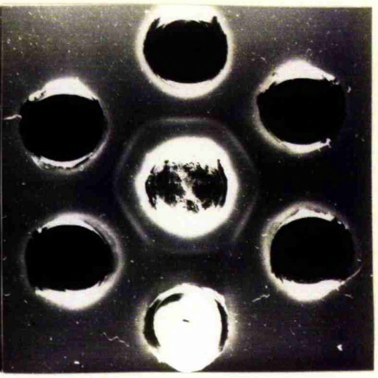

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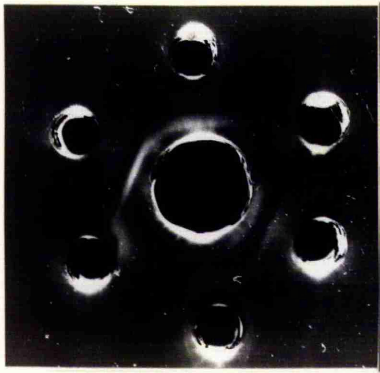

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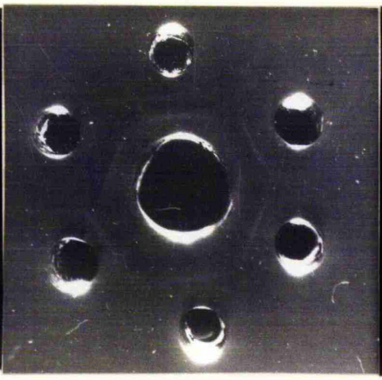

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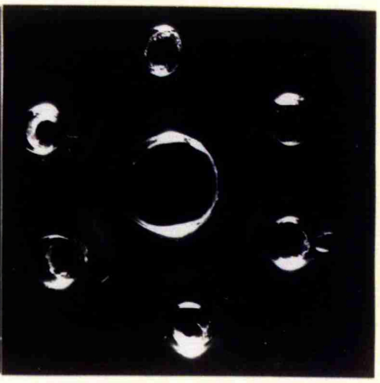

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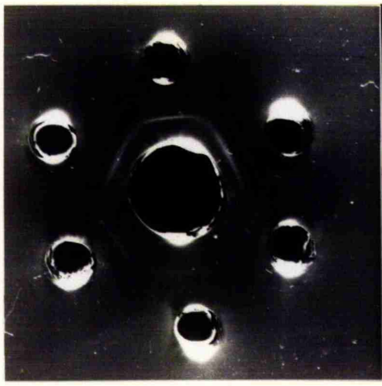

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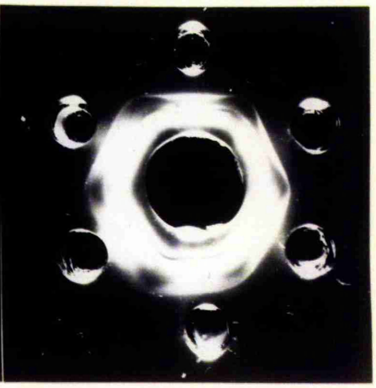

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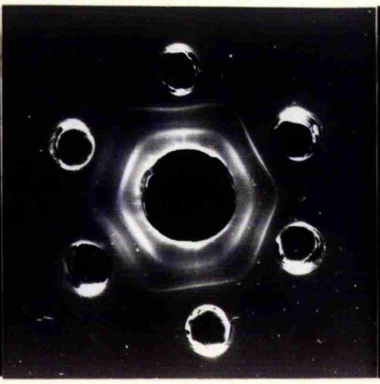

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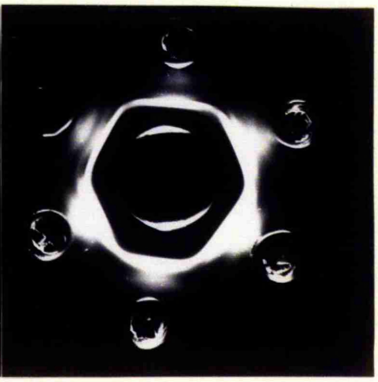

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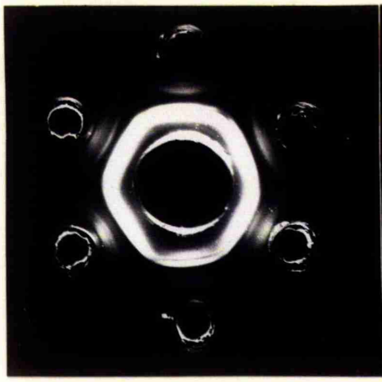

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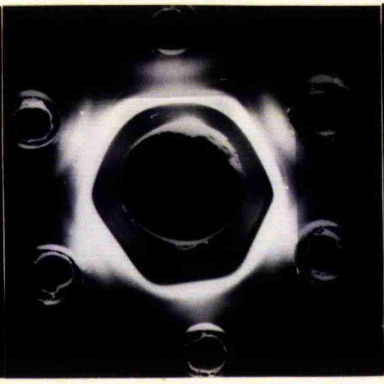

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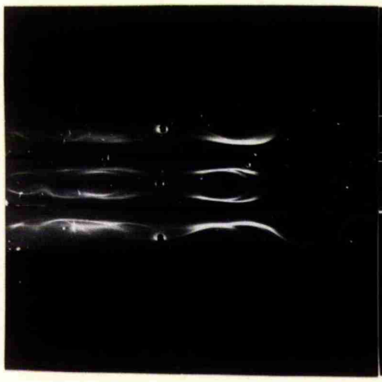

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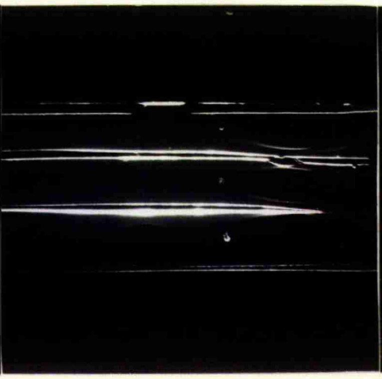

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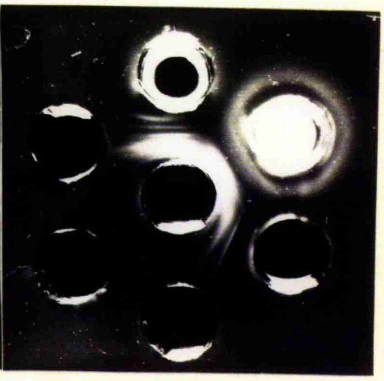

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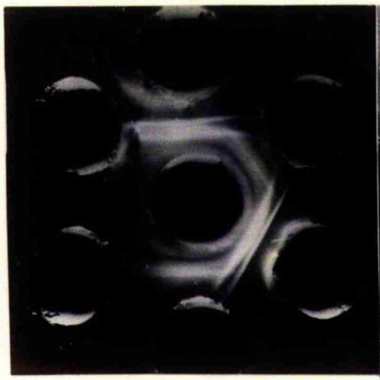

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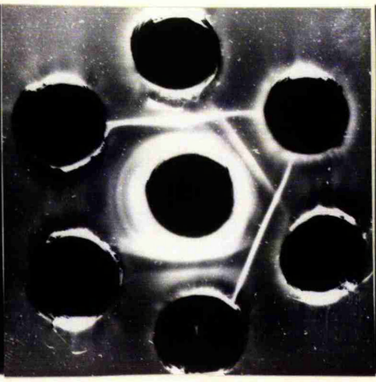

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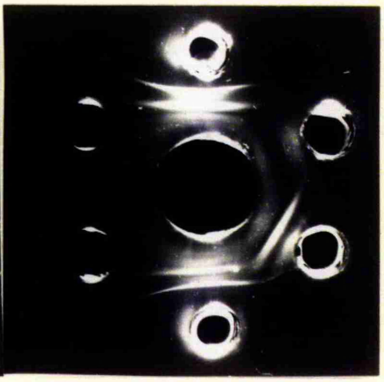

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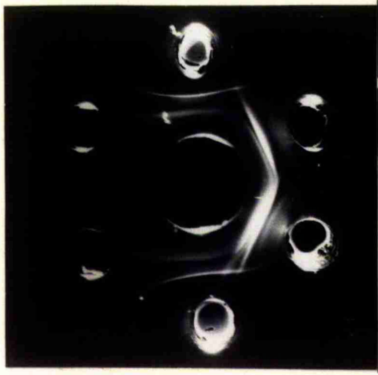

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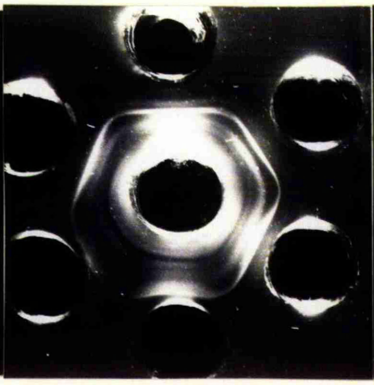

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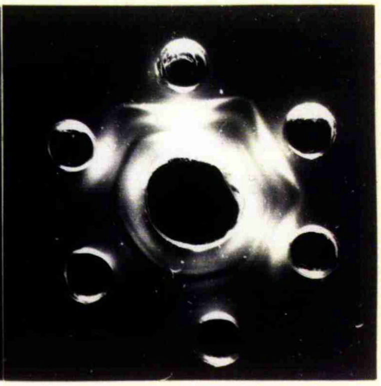

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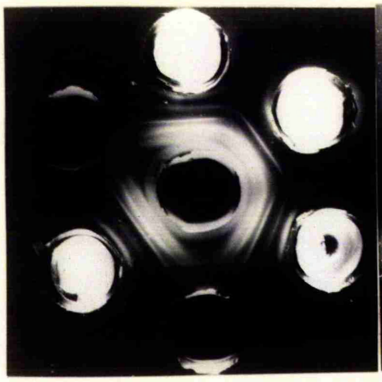

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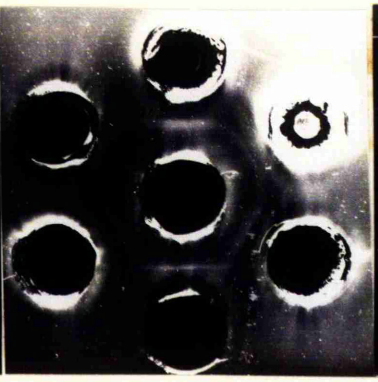

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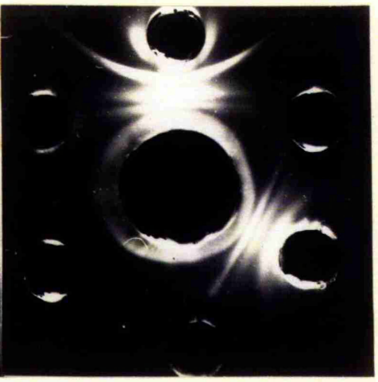

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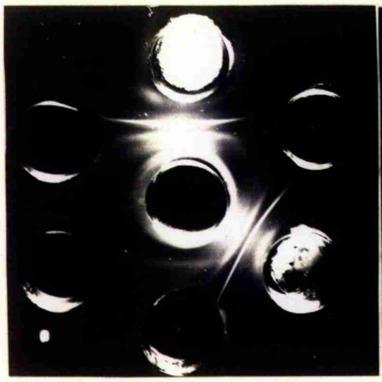

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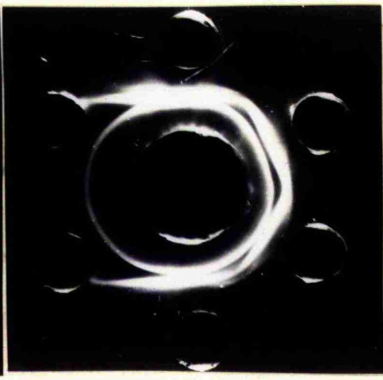

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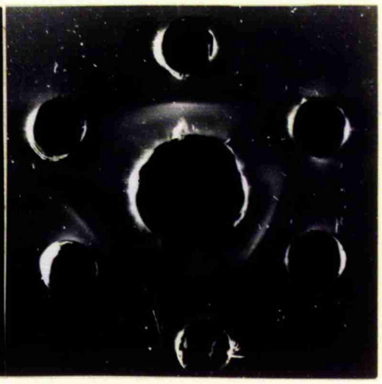

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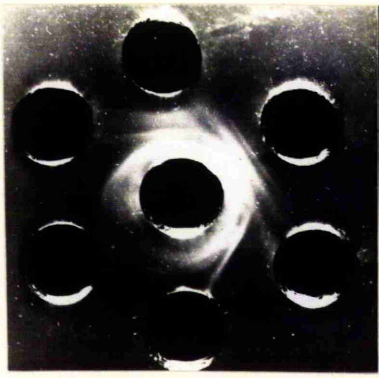

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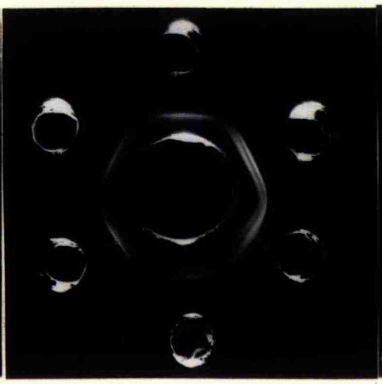

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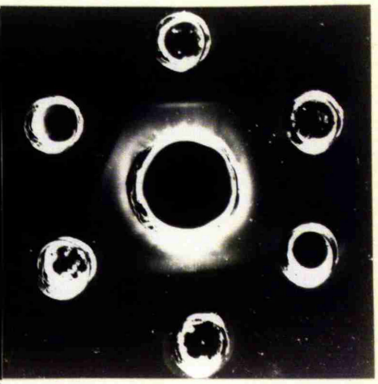

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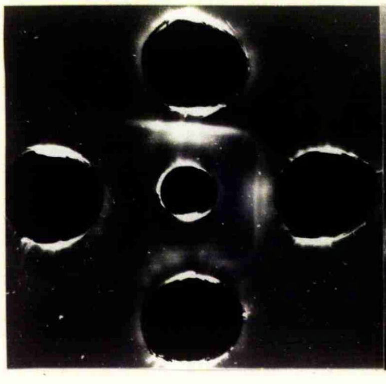

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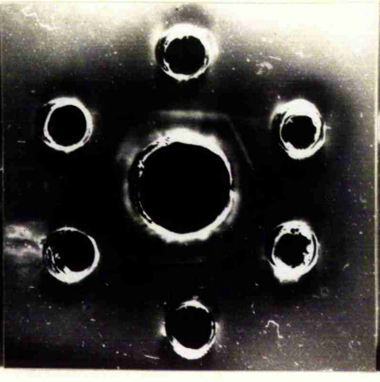

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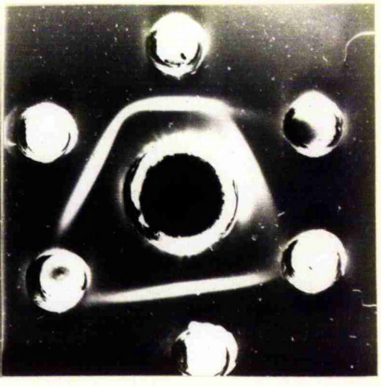

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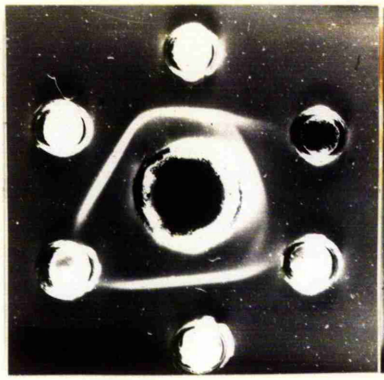

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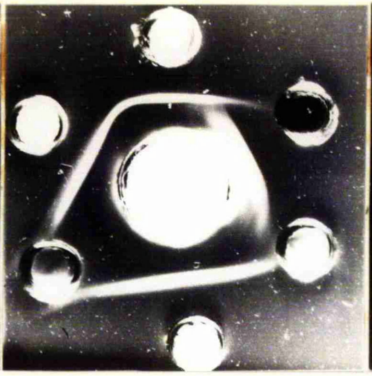

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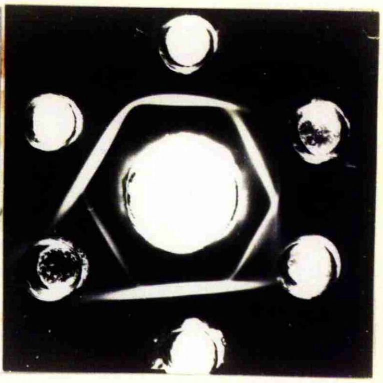

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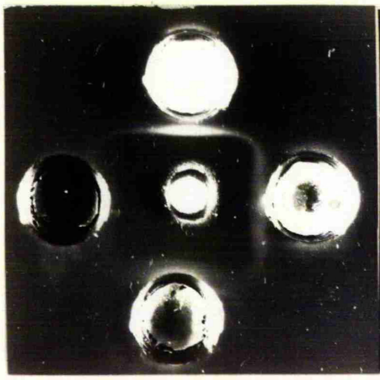

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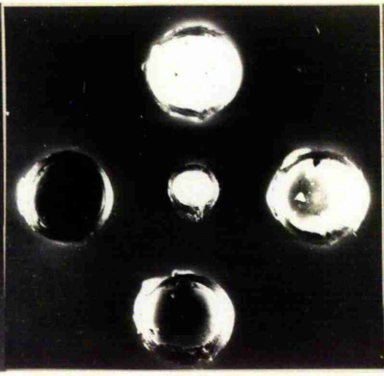

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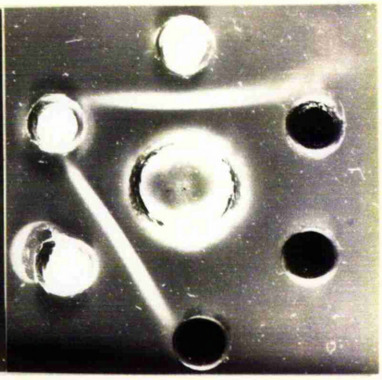

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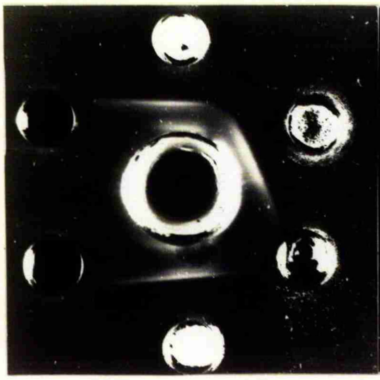

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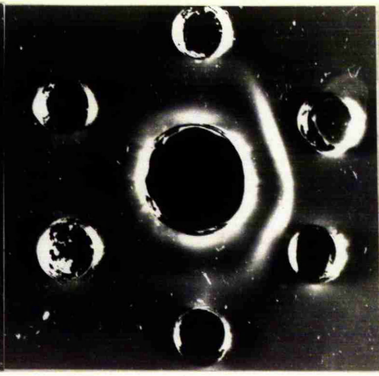

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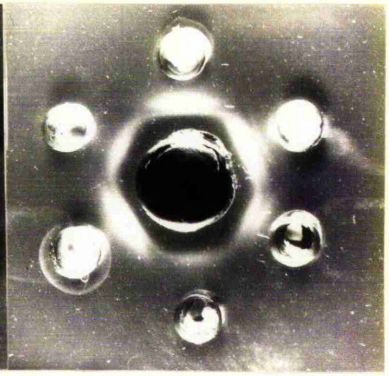

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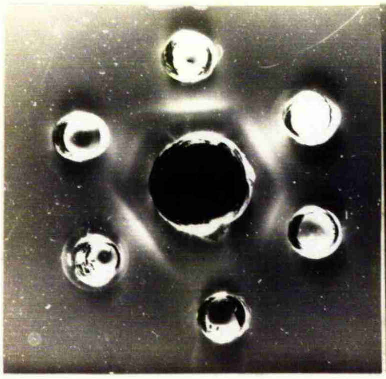

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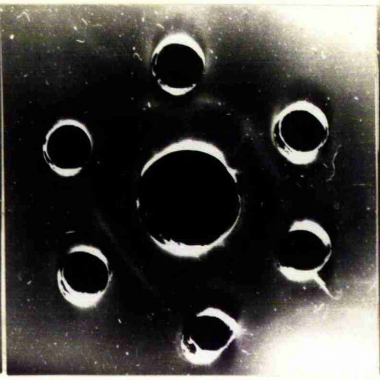

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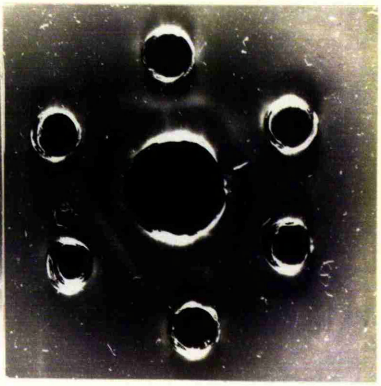

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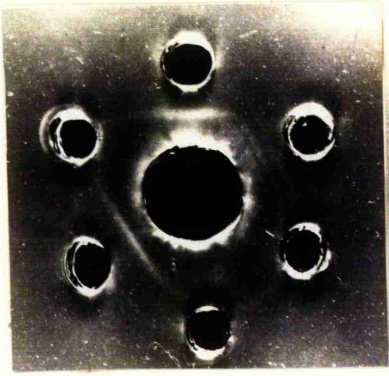

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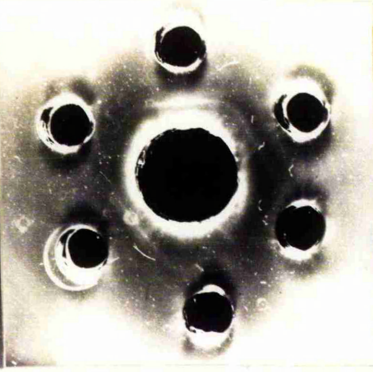

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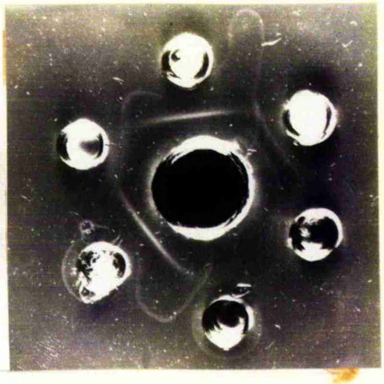

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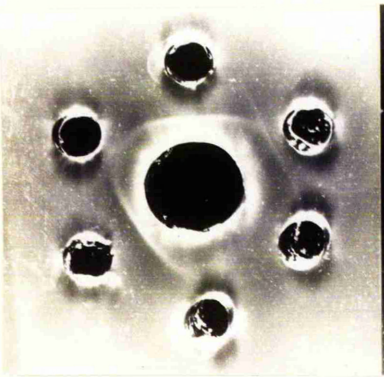

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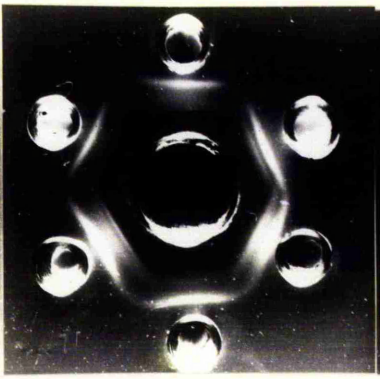

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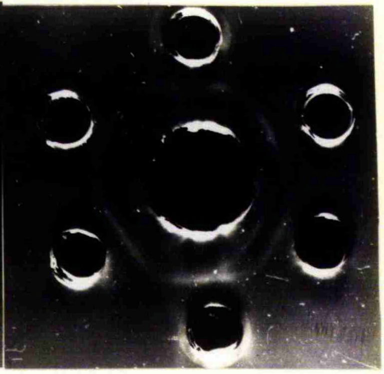

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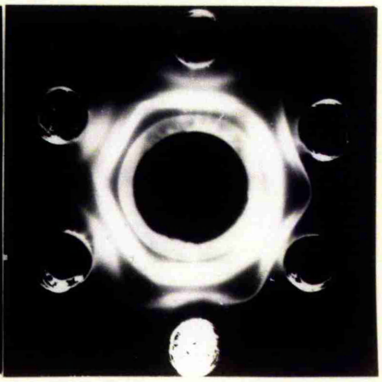

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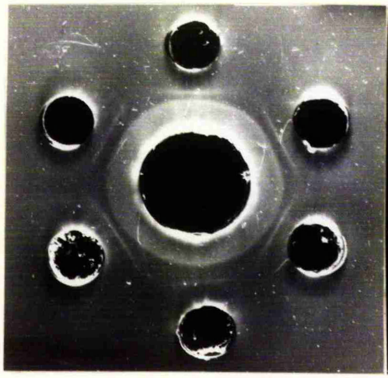

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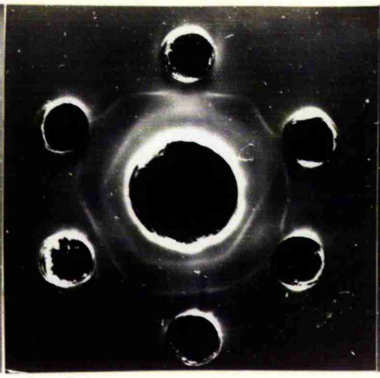

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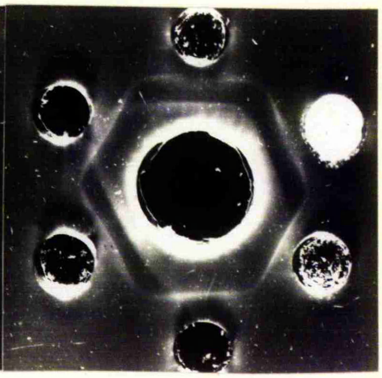

96

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